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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
UNDER 37 CFR 1.53(b)

Docket No.: 600-1-179N CON	Anticipated Classification of this Application: Class Subclass	Prior Application: Examiner: E. Stole	Art Unit: 1653
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Address to: ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, DC 20231

Sir:

This is a Request for Filing a ☒ Continuation ☐ Divisional Application under 37 CFR 1.53(b) of pending Application Serial No. 09/057,416, filed on April 8, 1998, of Olga Yurieva et al., for ENZYLE DERIVED FROM THERMOPHILIC ORGANISMS THAT FUNCTIONS AS A CHROMOSOMAL REPLICASE, AND PREPARATION AND USES THEREOF.

1. ☒ Enclosed is a copy of the prior application, including the oath or declaration as originally filed and an affidavit or declaration verifying it as a true copy.

2. ☐ Prepare a copy of the prior application.

3. ☒ A Filing Date as of the date of deposit in Express Mail is requested. The particulars of the Express Mail Deposit under 37 C.F.R. 1.10(b) are presented below.

EXPRESS MAIL "MAILING LABEL NO." : EL629424206US
DATE OF DEPOSIT : AUGUST 18, 2000

4. a. ☒ This is an application of a small entity under 37 CFR 1.9(f), and the amounts shown below in parentheses apply. A copy of the verified statement(s) filed in the prior application is enclosed.

b. ☐ The Filing Fee is calculated below:


FOR:	NO. FILED	NO. EXTRA	RATE	BASIC FEE
BASIC FEE				\$690.00
TOTAL CLAIMS	73 -20=	53	X\$18 (\$9)	\$
INDEP. CLAIMS	9 - 3=	6	X\$78 (\$39)	\$
TOTAL FILING FEE				
<input type="checkbox"/> MULTIPLE DEPENDENT CLAIM(S) PRESENTED (IF APPLICABLE)			X\$260.	\$
TOTAL OF ABOVE CALCULATIONS				\$
Reduction by 1/2 for filing by Small Entity (Note 37 CFR 1.3, 1.27, 1.28) If applicable, verified statement must be attached				
			TOTAL	\$

5. ☐ The Commissioner is hereby authorized to charge any fees which may be required in addition to those calculated above, or credit any overpayment to Deposit Account No. 11-1153.
6. ☐ A check in the amount of \$__ is enclosed.
7. ☐ Cancel in this application original Claims __ of the prior application before calculating the Filing Fee.
8. ☒ Amend the specification by inserting before the first line the sentence:
This Application is a ☒ Continuation ☐ Division, of Application Serial No. 09/057,416,
filed April 8, 1998.
9. ☐ Transfer the drawings from the prior application to this application and abandon said prior application as of the Filing Date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file.
10. ☒ New Formal Drawings are enclosed.
11. ☐ Priority of Application Serial No. _____, filed on _____ in _____ is claimed under 35 U.S.C. §119.
12. ☐ The certified copy has been filed in prior application Serial No. _____, filed _____.
13. ☒ The prior application is assigned of record to The Rockefeller University.
14. ☒ The power of attorney in the prior application is to:
Jackson Matalon (Attorney, Registration No. 22,441); Stefan J. Klauber (Attorney, Registration No. 22,604); David A. Jackson (Attorney, Registration No. 26,742); Michael D. Davis (Attorney, Registration No. 39,161); William C. Coppola (Attorney, Registration No. 41,687); Mark S. Cohen (Attorney, Registration No. 42,425); and Christine E. Dietzel (Agent, Registration No. 37,309).
- a. ☒ The power appears in the original papers in the prior application, a copy of which is enclosed.
- b. ☒ Associate Power of Attorney is enclosed.
- c. ☒ Address all future communications to:
David A. Jackson, Esq.
KLAUBER & JACKSON
411 Hackensack Avenue
Hackensack, NJ 07601.
15. ☐ A Preliminary Amendment is enclosed.
16. ☒ A copy of the Information Disclosure Statement from prior application. Pursuant to 37 C.F.R. 1.97(d), copies of the cited references are not enclosed.

17. [X] I hereby verify that the attached papers are a true copy of prior application Serial No. 09/057,416, as originally filed on April 8, 1998.
18. [X] I hereby state that the content of the paper and computer readable copies of the Sequence Listing submitted in accordance with 37 CFR §1.821(c) and (e), respectively, are the same.

The undersigned declares further that all statements made herein of his/her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: August 18, 2000


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Applicant or Patentee: Olga Yurieva et al

Application or Patent No.: 09/057,416

Filed or Issued: April 8, 1998

For: ENZYME DERIVED FROM THERMOPHILIC ORGANISMS THAT FUNCTIONS AS A CHROMOSOMAL REPLICASE, AND PREPARATION AND USES THEREOF

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 C.F.R. §§ 1.9(f) AND 1.27(d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION THE ROCKEFELLER UNIVERSITY

ADDRESS OF ORGANIZATION 1230 YORK AVENUE, NEW YORK, NEW YORK 10021-6399

TYPE OF ORGANIZATION

- ☒ University or other institution of higher education
☐ Tax exempt under Internal Revenue Service Code (26 U.S.C. §§ 501(a) and 501(c)(3))
☐ Nonprofit scientific or educational under statute of state of The United States of America
(Name of state _____)
(Citation of statute _____)
☐ Would qualify as tax exempt under Internal Revenue Service Code (26 U.S.C. §§ 501(a) and 501(c)(3)) if located in The United States of America
☐ Would qualify as nonprofit scientific or educational under statute of The United States of America if located in The United States of America
(Name of state _____)
(Citation of statute _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. § 1.9(e) for purposes of paying reduced fees under Sections 41(a) and 41(b) of Title 35, United States Code, with regard to the invention entitled by inventor(s) described in

- ☐ the specification filed herewith
☒ Application No. 09/057,416, filed APRIL 8, 1998
☐ Patent No. _____, issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the above-identified nonprofit organization are not exclusive, each individual, concern, or organization having rights to the invention is listed below,* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an individual inventor under 37 C.F.R. § 1.9(c), or by any concern that would not qualify as either a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

[illegible]

☐ individual ☐ small business concern ☐ nonprofit organization

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

ADDRESS OF PERSON SIGNING 1230 YORK AVENUE, NEW YORK, NY 10021-6399

DATE 5/17/88

**ENZYME DERIVED FROM THERMOPHILIC ORGANISMS THAT
FUNCTIONS AS A CHROMOSOMAL REPLICASE, AND PREPARATION
AND USES THEREOF**

FIELD OF THE INVENTION

- 5 The present invention relates to thermostable DNA polymerases, and more particularly to such polymerases as can serve as chromosomal replicases and are derived from thermophilic bacteria. More particularly, the invention extends to DNA polymerase III-type enzymes from thermophilic bacteria, including recombinant subunits thereof, to isolated DNA coding for such polymerases which hybridizes to
- 10 DNA probes prepared from the DNA sequence coding for *T. thermophilus* and its subunits, to DNA and antibody probes employed in isolation of said DNA, as well as to related methods for isolating said DNA and methods to express and purify the DNA and its subunits from the respective genes such as *dnaX*, *dnaA*, *dnaN*, *dnaQ*, *dnaE* and the like. The invention also relates to the purification and use of *T. thermophilus* Pol
- 15 III-type enzymes in efficient replication of a long natural template.

BACKGROUND OF THE INVENTION

- Thermostable DNA polymerases have been disclosed previously as set forth in U.S. Patent No. 5,192,674 to Oshima et al., U.S. Patent Nos. 5,322,785 and 5,352,778 to Comb et al., and U.S. Patent No. 5,545,552, and others. All of the noted references
- 20 recite the use of polymerases as important catalytic tools in the practice of molecular cloning techniques such as polymerase chain reaction (PCR). Each of the references states that a drawback of the extant polymerases are their limited thermostability, and consequent useful life in the participation in PCR. Such limitations also manifest themselves in the inability to obtain extended lengths of nucleotides, and in the
- 25 instance of Taq polymerase, the lack of 3' to 5' exonuclease activity, and the drawback of the inability to excise misinserted nucleotides (Tindall, et al. (1990) *Biochemistry* 29:5226-5231).

More generally, such polymerases, including those disclosed in the referenced patents, are of the Polymerase I variety as they have are approximately 90-95kDa in size and may have 5' to 3' exonuclease activity. They define a single subunit with concomitant limits on their ability to hasten the amplification process and to promote the rapid
 5 preparation of longer strands of DNA.

Chromosomal replicases are composed of several subunits in all organisms (Kornberg and Baker, 1992). In keeping with the need to replicate long chromosomes, replicases are rapid and highly processive multiprotein machines. All cellular replicases examined to date derive their processivity from one subunit that is shaped like a ring
 10 and completely encircles DNA (Kuriyan and O'Donnell, 1993; Kelman and O'Donnell, 1994). This "sliding clamp" subunit acts as a mobile tether for the polymerase machine (Stukenberg et. al., 1991). The sliding clamp does not assemble onto the DNA by itself, but requires a complex of several proteins, called a "clamp loader" which couples ATP hydrolysis to the assembly of sliding clamps onto DNA
 15 (O'Donnell et. al., 1992). Hence, cellular replicases are classically comprised of three components: a clamp, a clamp loader, and the DNA polymerase, and for purposes of the present invention, the foregoing components also serve as a broad definition of a "Pol III-type enzyme".

DNA polymerase III holoenzyme (Pol III holoenzyme) is the multi-subunit replicase
 20 of the *E. coli* chromosome. Pol III holoenzyme is distinguished from Pol I type DNA polymerases by its high processivity (>50 kbp) and rapid rate of synthesis (750 nts/s) (reviewed in Kornberg and Baker, 1991; Kelman and O'Donnell, 1995). The high processivity and speed is rooted in a ring shaped subunit, called β , that encircles DNA and slides along it while tethering the Pol III holoenzyme to the template (Stukenberg
 25 et. al., 1991; Kong et. al., 1992). The ring shaped β clamp is assembled around DNA by the multisubunit clamp loader, called γ complex. The γ complex couples the energy of ATP hydrolysis to the assembly of the β clamp onto DNA. This γ complex clamp loader is an integral component of the Pol III holoenzyme particle. A brief

overview of the organization of subunits within the holoenzyme and their function follows.

Pol III holoenzyme consists of 10 different subunits, some of which are present in multiple copies for a total of 18 polypeptide chains (Onrust et. al., 1995b). The organization of these subunits in the holoenzyme particle is illustrated in Fig. 1. As depicted in the diagram, the subunits of the holoenzyme can be grouped functionally into three components: 1) the DNA polymerase III core is the catalytic unit and consists of the α (DNA polymerase), ϵ (3'-5' exonuclease) and θ subunits (McHenry and Crow, 1979), 2) the β "sliding clamp" is the ring shaped protein that secures the core polymerase to DNA for processivity (Kong et. al., 1992), and 3) the 5 protein γ complex ($\gamma\delta\delta'\chi\psi$) is the "clamp loader" that couples ATP hydrolysis to assembly of β clamps around DNA (O'Donnell, 1987; Maki and Kornberg, 1988). A dimer of the τ subunit acts as a "macromolecular organizer" holding together two molecules of core and one molecule of γ complex forming the Pol III* subassembly (Onrust et. al., 1995b). This organizing role of τ to form Pol III* is indicated in the center of Fig. 1. Two β dimers associate with the two cores within Pol III* to form the holoenzyme capable of replicating both strands of duplex DNA simultaneously (Maki et. al., 1998).

The DNA polymerase III holoenzyme assembles onto a primed template in two distinct steps. In the first step, the γ complex assembles the β clamp onto the DNA. The γ complex and the core polymerase utilize the same surface of the β ring and they cannot both utilize it at the same time (Naktinis et. al., 1996). Hence, in the second step the γ complex moves away from β thus allowing access of the core polymerase to the β clamp for processive DNA synthesis. The γ complex and core remain attached to each other during this switching process by the τ subunit organizer.

The γ complex consists of 5 different subunits ($\gamma_{2-4}\delta_1\delta'_1\chi_1\psi_1$). An overview of the mechanism of the clamp loading process follows. The δ subunit is the major touch

5 DNA dependent ATPase activity and is competent to assemble clamps on DNA (Onrust et. al., 1991). Upon binding of ATP to γ , a change in the conformation of the complex exposes δ for interaction with β (Naktinis et. al., 1995). The function of the smaller subunits, χ and ψ , is to contact SSB (through χ) thus promoting clamp assembly and high processivity during replication (Kelman and O'Donnell, 1995).

10 The three component Pol III-type enzyme in eukaryotes contains a clamp that has the same shape as *E. coli* β , but instead of a homodimer it is a heterotrimer. This heterotrimeric ring, called PCNA (proliferating cell nuclear antigen), has 6 domains like β , but instead of each PCNA monomer being composed of 3 domains and dimerizing to form a 6 domain ring (e.g. like β), the PCNA monomer has 2 domains and it trimerizes to form a 6 domain ring (Krishna et. al., 1994; Kuriyan and O'Donnell, 1993). The chain fold of the domains are the same in prokaryotes (β) and eukaryotes (PCNA) and thus the rings have the same overall 6-domain ring shape. The clamp loader of the eukaryotic Pol III-type replicase is called RFC (Replication factor C) and it consists of subunits having homolgy to the γ and δ' subunits of the *E. coli* γ complex.

20 The eukaryotic DNA polymerase III-type enzyme contains either of two DNA polymerases, DNA polymerase δ and DNA polymerase ϵ . It is entirely conceivable that yet other types of DNA polymerases can function with either a PCNA or β clamp to form a Pol III-type enzyme (for example, DNA polymerase II of *E. coli* functions with the β subunit placed onto DNA by the γ complex clamp loader).

25 The bacteriophage T4 also utilizes a Pol III-type 3-component replicase. The clamp is a homotrimer like PCNA, called gene 45 protein. The gene 45 protein forms the same 6-domain ring structure as β and PCNA. The clamp loader is a complex of two subunits called the gene 44/62 protein complex. The DNA polymerase is the gene 43 protein and it is stimulated by the gene 45 sliding clamp when it is assembled onto

DNA by the 44/62 protein clamp loader. The Pol III-type enzyme may be either bound together into one particle (*e.g.*, *E. coli* Pol III holoenzyme), or its three components may not be assembled together into a stable particle in solution (like the eukaryotic Pol III-type replicases).

- 5 There is an early report on separation of three DNA polymerases from T.th. cells, however each polymerase form was reminiscent of the preexisting types of DNA polymerase isolated from thermophiles in that each polymerase was in the 110,000-120,000 range and lacked 3'-5' exonuclease activity (Ruttimann et. Al., 1985). These are well below the molecular weight of Pol III-type complexes that contain in addition
- 10 to the DNA polymerase subunit, other subunits such as γ and τ . Although the three polymerases displayed some differences in activity (column elution behavior, and optimum divalent cation, template, and temperatures) it seems likely that these three forms were either different repair type polymerases or derivatives of one repair enzyme (*e.g.* Pol I) that was modified into three forms by post translational
- 15 modification(s) that altered their properties (*e.g.* phosphorylation, methylation, slight proteolytic clipping of residues that alter activity, or association with different ligands such as a small protein or contaminating DNA). Despite this previous work, it remained to be demonstrated that thermophiles harbor a Pol III-type enzyme that contain multiple subunits such as γ and/or τ , functioned with a sliding clamp
- 20 accessory protein, or could extend a primer over a long stretch of ssDNA. Ruttimann, C., Cotoras, M., Zaldivar, J., and Vicuna, R. (1986) DNA polymerases from the extremely thermophilic bacterium *Thermus thermophilus* HB-8. *European J. of Biochem.* 149, 41-46.

- Previously it was not known how thermophilic bacteria replicated - only Pol I's have
- 25 been reported. By distinction, chromosomal replicases such as Polymerase III identified in *E. coli*, if available in a thermostable bacterium, with all its accessory subunits, could provide a great improvement over the Polymerase I's, in that they are

generally much more efficient - about 5 times faster and much more highly processive. Hence, one may expect faster and longer chain production in PCR, and higher quality of DNA sequencing ladders. Clearly the ability to practice such synthetic techniques as PCR would be enhanced by these methods disclosed for how to obtain genes and subunits of DNA polymerase III holoenzyme from thermophilic sources.

SUMMARY OF THE INVENTION

In accordance with the present invention, DNA Polymerase III-type enzymes as defined herein are disclosed that may be isolated and purified from a thermophilic bacterial source, that can function as a chromosomal replicase, and that possesses all of the structural and processive advantages sought and recited above. More particularly, the invention extends to the Polymerase III-type enzymes derived from thermostable thermophilic bacteria that exhibit the ability to extend a primer over a long stretch of ssDNA at elevated temperature, the ability to be stimulated by a cognate sliding clamp of the type that is assembled on DNA by a 'clamp' loader (*e.g.* γ complex), have clamp loading sub-units that show DNA stimulated ATPase activity at elevated temperature and/or ionic strength, and have a DNA polymerase-associated 3'-5' exonuclease activity (*e.g.*, ϵ subunit). Representative thermophiles include polymerases isolated from the thermophilic bacteria *Thermus thermophilus* (*T.th.* polymerase), *Thermococcus litoralis* (*Tli* or VENTTM polymerase), *Pyrococcus furiosus* (*Pfu* or DEEPVENT polymerase), *Pyrococcus woosii* (*Pwo* polymerase) and other *Pyrococcus* species, *Bacillus sterothermophilus* (*Bst* polymerase), *sulfolobus acidocaldarius* (*Sac* polymerase), *thermoplasma acidophilum* (*Tac* polymerase), *Thermus favus* (*Tfl/Tub* polymerase), *Thermus ruber* (*Tru* polymerase), *Thermus brockianus* (DYNAZYMETM polymerase), *Thermotoga neapolitana* (*Tne* polymerase; See WO 96/10640), *Thermotoga maritima* (*Tma* polymerase; See U.S. Patent No. 5,374,553) and other species of the *Thermotoga* genus (*Tsp* polymerase) and *Methanobacterium thermoautotrophicum* (*Mth* polymerase). In a preferred

embodiment, the thermophilic comprise those of the *Thermus* and *Thermotoga* species, and particularly *T.th.*, and *Tne* and *Tma*.

A particular Polymerase III-type enzyme in accordance with the invention may include at least one of the following sub-units:

- 5 A. a γ subunit having an amino acid sequence selected from the formula set forth in SEQ ID NOS:4 and 5;
- B. a τ subunit having an amino acid sequence corresponding to the formula set forth in SEQ ID NO:2;
- C. a ϵ subunit having an amino acid sequence corresponding to the
- 10 formula set forth in SEQ ID NO:95;
- D. a α subunit including an amino acid sequence corresponding to the formula set forth in SEQ ID NO:87;
- E. a β subunit having an amino acid sequence corresponding to the formula set forth in SEQ ID NO:107; and
- 15 variants, including allelic variants, muteins, analogs and fragments of any of subparts (A) through (E), and combinations thereof, capable of functioning in DNA amplification and sequencing.

The invention also extends to the genes that correspond to and can code on expression for the subunits set forth above, and accordingly includes the following: *dnaX*, *dnaQ*,
20 *dnaE* and *dnaN*, and conserved variants and active fragments thereof.

Accordingly, the Polymerase III-type enzyme of the present invention comprises at least one gene encoding a subunit thereof, which gene is selected from the group consisting of *dnaX*, *dnaQ*, *dnaE* and *dnaN*, and combinations thereof. More particularly, the invention extends to the nucleic acid molecule encoding the γ and τ
25 subunits, and includes the *dnaX* gene which has a nucleotide sequence as set forth in SEQ ID NO. 3, as well as conserved variants, active fragments and analogs thereof. Likewise, the nucleotide sequences encoding the α subunit (*the dnaE* gene), the ϵ

subunit (*dnaQ* gene) and the β subunit (*dnaN* gene) each comprise the nucleotide sequences as set forth respectively, in SEQ ID NO'S: 94; 86 and 106, as well as conserved variants, active fragments and analogs thereof.

- The invention also provides methods and products for identifying, isolating and
 5 cloning DNA molecules which encode such accessory subunits encoded by the recited genes of the DNA polymerase III-type enzyme hereof.

- Yet further, the invention extends to Polymerase III-type enzymes prepared by the purification of an extract taken from *e.g.* the particular thermophile under examination, treated with appropriate solvents and then subjected to chromatographic
 10 separation on *e.g.* an anion exchange column, followed by analysis of long chain synthetic ability or Western analysis of the respective peaks against antibody to at least one of the anticipated enzyme subunits to confirm presence of Pol III, and thereafter, peptide sequencing of subunits that co purify and amplification to obtain the putative gene and its encoded enzyme.

- 15 The present invention also relates to recombinant γ , τ , ϵ , α and β subunits from thermophiles. In the instance of the γ and τ subunits, the invention includes the characterization of a frameshifting sequence that is internal to the gene and specifies relative abundance of the γ and τ gene products of *dnaX*. From this characterization it is obvious how to increase expression of either one of the subunits at the expense of
 20 the other (i.e. mutant frameshift could make all τ , simple recloning at the end of the frameshift could make exclusively γ and no τ).

- In a further aspect of the present invention, DNA probes can be constructed from the DNA sequences coding for, *eg.* the *T.th. dnaX*, *dnaQ*, *dnaE*, *dnaA* and *dnaN* genes, conserved variants and active fragments thereof, all as defined herein, and may be
 25 used to identify and isolate the corresponding genes coding for the subunits of DNA polymerase III holoenzyme from other thermophiles, such as those listed earlier

herein. Accordingly, all chromosomal replicases (DNA Polymerase III-type) from thermophilic sources are contemplated and included herein.

The invention also extends to methods for identifying Polymerase III-type enzymes by use of the techniques of long-chain extension and elucidation of subunits with
5 antibodies, as described herein and with reference to the examples.

The invention further extends to the isolated and purified DNA Polymerase III, the amino acid sequences of the γ , τ , ϵ , α and β subunits, as set forth in SEQ ID NOS:4, 5, 2, 95, 87, and 107. and the nucleotide sequences of the corresponding genes from *T.th.* set forth, *e.g.* in SEQ ID NOS:3 (*dnaX*), 94 (*dnaQ*), 86 (*dnaE*) and 106 (*dnaN*),
10 as well as to active fragments thereof, oligonucleotides and probes prepared or derived therefrom and the transformed cells that may be likewise prepared. Accordingly, the invention comprises the individual subunits enumerated above and hereinafter, corresponding isolated polynucleotides and respective amino acid sequences for each of the γ , τ , ϵ , α and β subunits, and to conserved variants, fragments, and the like, as
15 well as to methods of their preparation and use in DNA amplification and sequencing. In a particular embodiment, the invention extends to vectors for the expression of the sub-unit genes of the present invention. and more specifically to the vectors pET16*dnaX* and pET24*dnaN*.

The invention also includes methods for the preparation of the DNA Polymerase III-
20 type enzymes and the corresponding subunit genes of the present invention, and to the use of the enzymes and constructs having active fragments thereof, in the preparation, reconstitution of modification of like enzymes, as well as in amplification and sequencing of DNA by methods such as PCR, and like protocols, and to the DNA molecules amplified and sequenced by such methods. In this regard, a Pol III-type
25 enzyme that is reconstituted in the absence of ϵ , or using a mutated ϵ with less 3'-5' exonuclease activity, may be a superior enzyme in either PCR or DNA sequencing applications, (*e.g.* Tabor and Richardson, 1995.)

The invention is directed to methods for amplifying and sequencing a DNA molecule, particularly via the polymerase chain reaction (PCR), using the present DNA polymerase III-type enzymes or complexes. In particular, the invention extends to methods of amplifying and sequencing of DNA using thermostable pol III-type
5 enzyme complexes isolated from thermophilic bacteria such as *Thermotoga* and *Thermus* species, or recombinant thermostable enzymes. The invention also provides amplified DNA molecules made by the methods of the invention, and kits for amplifying or sequencing a DNA molecule by the methods of the invention.

In this connection, the invention extends to methods for amplification of DNA that
10 can achieve long chain extension of primed DNA, as by the application and use of Polymerase III-type enzymes of the present invention. An illustration of such methods is presented in Examples 13 and 14, *infra*.

Likewise, kits for amplification and sequencing of such DNA molecules are included, which kits contain the enzymes of the present invention, including subunits thereof,
15 together with other necessary or desirable reagents and materials, and directions for use. The details of the practice of the invention as set forth above and later on herein, and with reference to the patents and literature cited herein, are all expressly incorporated herein by reference and made a part hereof.

As stated, and in accordance with a principal object of the present invention,
20 Polymerase III-type enzymes and their sub-units are provided that are derived from thermophiles and that are adapted to participate in improved DNA amplification and sequencing techniques, and the consequent ability to prepare larger DNA strands more rapidly and accurately.

It is a further object of the present invention to provide DNA molecules that are
25 amplified and sequenced using the Polymerase III-type enzymes hereof.

It is a still further object of the present invention to provide enzymes and corresponding methods for amplification and sequencing of DNA that can be practiced without the participation of the clamp-loading component of the enzyme.

It is a still further object of the present invention to provide kits and other assemblies of materials for the practice of the methods of amplification and sequencing as aforesaid, that include and use the DNA polymerase III-type enzymes herein as part thereof.

Other objects and advantages will become apparent from a review of the ensuing description which proceeds with reference to the following illustrative drawings.

10

DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a schematic depiction of the structure and components of enzymes of the general family to which the enzymes of the present invention belong.

FIGURE 2. Alignment of the N-terminal regions of *E. coli* and *B. subtilis dnaX* gene product - Asterisks indicate identities. The ATP binding consensus sequence is indicated. The two regions used for PCR primer design are shown in bold.

15

FIGURE 3. Southern analysis of *T. thermophilus* genomic DNA - Genomic DNA was analyzed for presence of the *DnaZ* gene using the PCR radiolabelled probe. Enzymes used for digestion are shown above each lane. The numbering to the right corresponds to the length of DNA fragments (kb).

FIGURES 4A and 4B depict the full sequence of the *dnaX* gene of *T. thermophilus* - DNA sequence (upper case, and corresponding to SEQ ID NO:1) and predicted amino acid sequence (lower case, and corresponding to SEQ ID NO:2) yields a 529 amino acid protein (τ) of 58.0 kDa. A putative frameshifting sequence containing several A

20

- residues 1478-1486 (underlined) may produce a smaller protein (γ) of 49.8 kDa. The potential Shine-Dalgarno (S.D.) signal is bold and underlined. The start codon is in bold, and the stop codon for τ is marked by an asterisk. The potential stop codon for γ is shown in bold after the frameshift site, and two potential Shine-Dalgarno sequences upstream of the frameshift site are indicated. Sequences of the primers used for PCR are shown in italics above the nucleotide sequence of *dnaX*. The ATP binding site is indicated, and the asterisks above the four Cys residues near the ATP site indicate the putative Zn⁺⁺ finger. The proline rich area is indicated above the sequence. Numbering of the nucleotide sequence is presented to the right.
- 10 Numbering of the amino acid sequence of τ is shown in parenthesis to the right.

FIGURE 4C depicts the isolated DNA coding sequence for the *dnaX* gene (also present in FIGURES 3A and 3B) in accordance with the invention, and corresponds to SEQ ID NO:3.

- FIGURE 4D depicts the polypeptide sequence of the γ subunit of the Polymerase III of the present invention, and corresponds to SEQ ID NO:4.

FIGURE 4E depicts the polypeptide sequence of the γ subunit of the Polymerase III of the present invention defined by a -1 frameshift, and corresponds to SEQ ID NO:4.

- FIGURE 4F depicts the polypeptide sequence of the γ subunit of the Polymerase III of the present invention defined by a -2 frameshift, and corresponds to SEQ ID NO:5.

- FIGURE 5. Alignment of the γ/τ ATP binding domains for different bacteria - Dots indicate those residues that are identical to the *E. coli dnaX* sequence. The ATP consensus site is underlined, and the conserved cysteine residues that form the zinc finger are indicated with asterisks. *E. coli*, *Escherichia coli*; *H. inf.*, *Haemophilus influenzae*; *B. sub.*, *Bacillus subtilis*; *C. cres.*, *Caulobacter crescentus*; *M. gen.*,

Mycoplasma genitalium; *T.th.*, *Thermus thermophilus*. Alignments were produced using Clustel.

FIGURE 6. Signal for ribosomal frameshifting in *T.th. dnaX* - The diagram shows part of the sequence of the RNA around the frameshifting site, including the suspected
 5 slippery sequence A9 (bold italic). The stop codon in the -2 reading frame is indicated. Also indicated are potential step loop structures and the nearest stop codons in the -1 reading frame.

FIGURE 7. Analysis of γ and τ in *T.th.* cells by Western - Whole cells were lysed in SDS and electrophoresed on a 10 % SDS polyacrylamide gel then transferred to a
 10 membrane and probed with polyclonal antibody against *E. coli* γ/τ as described in Experimental Procedures. Positions of molecular weight size markers are shown to the left. Putative *T.th.* γ and τ are indicated to the right.

FIGURE 8. The frameshift sequence in *T.th. dnaX* promotes -1 and -2 frameshifts in *E. coli* - The region of the *dnaX* gene slippery sequence was cloned into the lacZ gene
 15 of pUC19 in three reading frames, then transformed into *E. coli* cells and plated on LB plates containing X-gal. The slippery sequence was also mutated by inserting two G residues into the A9 sequence and then cloned into pUC19 in all three reading frames. Color of colonies observed are indicated by the plus signs. The picture shows the colonies, the type of frameshift required for readthrough (blue color) is
 20 indicted next to the sector.

FIGURE 9. Construction of the *T.th.* γ/τ expression vector - A genomic fragment containing a partial sequence of *dnaX* was cloned into pALTER-1. This fragment was subcloned into pUC19 (pUC19_*dnaX*). Then the N-terminal section of *dnaX* was amplified such that the fragment was flanked by NdeI (at the initiating codon) and the
 25 internal BamHI site. This fragment was inserted to form the entire coding sequence of the *dnaX* gene in pUC19 (pUC19*dnaX*). The *dnaX* gene was then cloned behind the

polyhistidine leader in the T7 based expression vector pET16 to give pET16*dnaX*. Details are in "Experimental Procedures".

FIGURE 10. Purification of recombinant *T.th.* γ and τ subunits - *T.th.* γ and τ subunits were expressed in *E. coli* harboring pET16*dnaX*. Molecular size markers are shown to the left of the gels, and the two induced proteins are labelled as g and t to the right of the gel. Panel A) 10% SDS gel of *E. coli* whole cell lysates before and after induction with IPTG. Panel B) 8% SDS gel of the purification two steps after cell lysis. First lane: the lysate was applied to a HiTrap Nickel chromatography column. Second lane: the *T.th.* γ/τ subunits were further purified on a Superose 12 gel filtration column. Third lane, the *E. coli* γ and τ subunits. Panel C) Western analysis of the pure *T.th.* γ and τ subunits (first lane) and *E. coli* γ and τ subunits (second lane).

FIGURE 11. Gel filtration of *T.th.* γ and τ - *T.th.* γ and τ were gel filtered on a Superose 12 column. Column fractions were analyzed for ATPase activity and in a Coomassie Blue stained 10% SDS polyacrylamide gel. Positions of molecular weight markers are shown to the left of the gel. The elution position of size standards analyzed in a parallel Superose 12 column under identical conditions are indicated above the gel. Thyroglobin (670 kDa), bovine gamma globin (150 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa).

FIGURE 12. Characterization of the *T.th.* γ and τ ATPase activity - The *T.th.* γ/τ and *E. coli* τ subunits are compared in their ATPase activity characteristics. Due to the greater activity of *E. coli* τ , the values are plotted as percent for ease of comparison. Actual specific activities for 100 % values are given below as pmol ATP hydrolyzed/30 min./pmol *T.th.* γ/τ (or pmol *E. coli* τ). Panel A) *T.th.* γ and τ ATPase is stimulated by the presence of ssDNA. *T.th.* γ/τ was incubated at 65°C. Specific activity was: 11.5 (+DNA); 2.5 (-DNA); *E. coli* τ was assayed at 37°C. Specific activity values were: 112.5 (+DNA); (7.3-DNA). Panel B) Temperature stability of

DNA stimulated ATPase activity. *T.th.* γ/τ , 11.3 (65°C); *E. coli* τ , 97.5 (37°C).

Panel C) Stability of *T.th.* γ/τ ATPase to NaCl. *T.th.* γ/τ , 8.1 (100 mM added NaCl and 65°C); *E. coli* τ , 52.7 (0 M added NaCl and 37°C).

FIGURES 13A-13C are graphs that summarize the purification of the DNA

- 5 polymerase III from *T.th.* extracts. A) shows the activity and total protein in column fractions from the Heparin Agarose column. Peak 1 fractions were chromatographed on ATP agarose, and Panel B) depicts the ATP-agarose column step, and Panel C) shows the total protein and DNA polymerase activity eluted from the MonoQ column.

FIGURE 14 is a 12% SDS polyacrylamide gel stained with Coomassie Blue (Panel A)

- 10 of the MonoQ column. Loud stands for the material loaded onto the column (ATP agarose bound fractions). FT stands for protein that flowed through the MonoQ column. Fractions are indicated above the gel. *T.th.* subunits α , τ , γ , δ , δ' in fractions 17-19 are indicated by the labels placed between fractions 18 and 19. Additional small subunits may be present but difficult to visualize, or may have run off the gel.
- 15 *E. Coli*, γ , δ shows a mixture of the α , γ and δ subunits of DNA polymerase III holoenzyme (they are labelled to the right in the figure). Panel B shows the Western results of an SDS gel of the MonoQ fractions probed with rabbit antiserum raised against the *E. coli* α subunit. L and FT are as described in Panel A. Fraction numbers are shown above the gel. The band that comigrates with *E. coli* α , and the band in the
- 20 Coomassie Blue stained gel in Panel A, is marked with an arrow. This band was analyzed for microsequence and the results are shown in Fig. 15.

FIGURE 15 shows the alignments of the peptides obtained from *T.th.* α subunit,

TTH1 (shown in A) and TTH2 (shown in B) with the amino acid sequences of the α subunits of other organisms. The amino acid number of these regions within each

- 25 respective protein sequence are shown to the right. The abbreviations of the organisms are as follows. *E.coli* - *Escherichia coli*, *V.chol.* - *Vibrio cholerae*, *H.inf.* - *Haemophilus influenzae*, *R.prow.* - *Rickettsia prowazekii*, *H.pyl.* - *Helicobacter*

pylori, *S.sp.* - *Synechocystis sp.*, *M.tub.* - *Mycobacterium tuberculosis*, *T.th.* - *Thermus thermophilus*.

FIGURE 16 shows a partial nucleotide (Panel A) and amino acid (Panel B) sequence of the *dnaE* gene encoding the α subunit of DNA polymerase III holoenzyme. The peptide sequence in bold was obtained by microsequencing of the α subunit isolated from *T.th.* cells.

FIGURE 17 shows an alignment of the amino acid sequence of ϵ subunits encoded by *dnaQ* of several organisms. The amino acid sequence of the *Thermus thermophilus* ϵ subunit of *dnaQ* is also shown. *T.th.*, *Thermus thermophilus*; *D.rad.*, *Deinococcus radiodurans*; *Bac.sub.*, *Bacillus subtilis*; *H.inf.*, *Haemophilus influenzae*; *E.c.*, *Escherichia coli*; *H.pyl.*, *Helicobacter pylori*. The regions used to obtain the inner part of the *dnaQ* gene are shown in bold. The starts used for expression of the *T.th.* ϵ subunit are marked.

FIGURE 18 shows the nucleotide (Panel A) and amino acid (Panel B) sequence of the *dnaQ* gene encoding the ϵ subunit of DNA polymerase III holoenzyme.

FIGURE 19 shows an alignment of the DnaA protein of several organisms. The amino acid sequence of the *Thermus thermophilus* DnaA protein of is also shown. *T.th.*, *Thermus thermophilus*; *Bac.sub.*, *Bacillus subtilis*; *E.c.*, *Escherichia coli*; *H.pyl.*, *Helicobacter pylori*; *M. tub.*, *Mycobacterium tuberculosis*; *T. mar.*, *Thermatoga maritima*.

FIGURE 20 shows the nucleotide (Panel A) and amino acid (Panel B) sequence of the *dnaA* gene of *Thermus thermophilus*.

FIGURE 21 shows the nucleotide (Panel A) and amino acid (Panel B) sequence of the *dnaN* gene encoding the β subunit of DNA polymerase III holoenzyme.

[illegible]

27

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(i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 27 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO
```

27

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(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "PRIMER"

iii) HYPOTHETICAL: NO
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30

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(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 23 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "PRIMER"

ii) HYPOTHETICAL: NO
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FIGURE 26 shows the use of *T.th.* Pol III in extending singly primed M13mp18 to an RFII form. The scheme at the top shows the primed template in which a DNA 57mer was annealed to the M13mp18 ssDNA circle. Then *T.th.* β subunit (produced recombinantly) and *T.th.* Pol III were added to the DNA in the presence of radioactive nucleoside triphosphates. In panel B, the products of the reaction were analyzed in a 0.8% native agarose gel. The position of ssDNA starting material, the RFII product, and of intermediate species, are shown to the sides of the gel. Lane 1, use of Pol III from the Heparin Agarose peak 1. Lane 2, use of the non-Pol III DNA polymerase contained in the peak 2 of the *T.th.* Heparin Agarose column.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

The terms "DNA Polymerase III," "Polymerase III-type enzyme(s)", "Polymerase III enzyme complex(s)", "*T.th.* DNA Polymerase III", "clamp loader" and any variants not specifically listed, may be used herein interchangeably, as are β subunit and

- sliding clamp and clamp as are also γ complex, clamp loader and RFC, as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in the Figures and corresponding
- 5 Sequence Listing entries, and the corresponding profile of activities set forth herein and in the Claims. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the
- 10 complex or its named subunits. Also, the terms "DNA Polymerase III," "*T.th.* DNA Polymerase III," and " γ and τ subunits", " β subunit", " α subunit", " ϵ subunit", "sliding clamp" and "clamp loader" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.
- 15 Also as used herein, the term "thermolabile enzyme" refers to a DNA polymerase which is not resistant to inactivation by heat. For example, T5 DNA polymerase, the activity of which is totally inactivated by exposing the enzyme to a temperature of 90°C for 30 seconds, is considered to be a thermolabile DNA polymerase. As used herein, a thermolabile DNA polymerase is less resistant to heat inactivation than in a
- 20 thermostable DNA polymerase. A thermolabile DNA polymerase typically will also have a lower optimum temperature than a thermostable DNA polymerase. Thermolabile DNA polymerases are typically isolated from mesophilic organisms, for example mesophilic bacteria or eukaryotes, including certain animals.
- 25 As used herein, the term "thermostable enzyme" refers to an enzyme which is stable to heat and is heat resistant and catalyzes (facilitates) combination of the nucleotides in the proper manner to form the primer extension products that are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each

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primer and will proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths.

The thermostable enzyme herein must satisfy a single criterion to be effective for the amplification reaction, i.e., the enzyme must not become irreversibly denatured

- 5 (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded nucleic acids. Irreversible denaturation for purposes herein refers to permanent and complete loss of enzymatic activity. The heating conditions necessary for denaturation will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the nucleic acids being
- 10 denatured, but typically range from about 90° to about 96°C for a time depending mainly on the temperature and the nucleic acid length, typically about 0.5 to four minutes. Higher temperatures may be tolerated as the buffer salt concentration and/or GC composition of the nucleic acid is increased. Preferably, the enzyme will not become irreversibly denatured at about 90°-100°C.
- 15 The thermostable enzymes herein preferably have an optimum temperature at which they function that is higher than about 40°C, which is the temperature below which hybridization of primer to template is promoted, although, depending on (1) magnesium and salt concentrations and (2) composition and length of primer, hybridization can occur at higher temperature (e.g., 45°-70°C). The higher the
- 20 temperature optimum for the enzyme, the greater the specificity and/or selectivity of the primer-directed extension process. However, enzymes that are active below 40°C, e.g., at 37°C, are also within the scope of this invention provided they are heat-stable. Preferably, the optimum temperature ranges from about 50° to 90°C, more preferably 60°-80°C. In this connection, the term "elevated temperature" as used herein is
- 25 intended to cover sustained temperatures of operation of the enzyme that are equal to or higher than about 60°C.

The term "template" as used herein refers to a double-stranded or single-stranded DNA molecule which is to be amplified, synthesized or sequenced. In the case of a double-stranded DNA molecule, denaturation of its strands to form a first and a second strand is performed before these molecules may be amplified, synthesized or sequenced. A primer, complementary to a portion of a DNA template is hybridized under appropriate conditions and the DNA polymerase of the invention may then synthesize a DNA molecule complementary to said template or a portion thereof. The newly synthesized DNA molecule, according to the invention, may be equal or shorter in length than the original DNA template. Mismatch incorporation during the synthesis or extension of the newly synthesized DNA molecule may result in one or a number of mismatched base pairs. Thus, the synthesized DNA molecule need not be exactly complementary to the DNA template.

The term "incorporating" as used herein means becoming a part of a DNA molecule or primer.

As used herein "amplification" refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence, or its complimentary sequence, with the use of a DNA polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA molecule or primer thereby forming a new DNA molecule complementary to a DNA template. The formed DNA molecule and its template can be used as templates to synthesize additional DNA molecules. As used herein, one amplification reaction may consist of many rounds of DNA replication. DNA amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 30 to 100 "cycles" of denaturation and synthesis of a DNA molecule. In this connection, the use of the term "long stretches of DNA" as it refers to the extension of primer along DNA is intended to cover such extensions of an average length exceeding 7 kilobases. Naturally, such length will vary, and all such variations are considered to be included within the scope of the invention.

[illegible]

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

Arg Val Arg Asp Gly Leu Leu Glu Ile Gly Arg Ala Leu Asn Ile Pro
20 25 30

(2) INFORMATION FOR SEQ ID NO:79:

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Ile Pro Leu Asp Asp Lys Lys Ser Phe Asp Met Leu Gln Arg Ser Glu
20 25 30

Thr Thr Ala Val Phe Gln Leu Glu Ser Arg Gly Met Lys Asp
35 40 45

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

nomenclature, *J. Biol. Chem.*, **243**:3552-59 (1969). abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

<u>SYMBOL</u>		<u>AMINO ACID</u>	
	<u>1-Letter</u>	<u>3-Letter</u>	
5	Y	Tyr	tyrosine
	G	Gly	glycine
	F	Phe	phenylalanine
	M	Met	methionine
10	A	Ala	alanine
	S	Ser	serine
	I	Ile	isoleucine
	L	Leu	leucine
	T	Thr	threonine
15	V	Val	valine
	P	Pro	proline
	K	Lys	lysine
	H	His	histidine
	Q	Gln	glutamine
20	E	Glu	glutamic acid
	W	Trp	tryptophan
	R	Arg	arginine
	D	Asp	aspartic acid
	N	Asn	asparagine
25	C	Cys	cysteine

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the

beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions
5 as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine,
10 guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-
15 stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

An "origin of replication" refers to those DNA sequences that participate in DNA
20 synthesis.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl)
25 terminus. A coding sequence can include, but is not limited to, prokaryotic

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000000 000000

sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

- 5 Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding
 10 sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence
 15 will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An "expression control sequence" is a DNA sequence that controls and regulates the
 20 transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

A "signal sequence" can be included before the coding sequence. This sequence
 25 encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the

media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

5 The term "oligonucleotide," as used generally herein, such as in referring to probes prepared and used in the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

10 The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either
15 single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer
20 typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the
25 primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand.

Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

- 5 As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

- A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be
 10 integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through
 15 chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

- 20 Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization
 25 experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art.

See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

It should be appreciated that also within the scope of the present invention are DNA sequences encoding *T.th.* DNA Polymerase III which code for a *T.th.* DNA

- 5 Polymerase III having the same amino acid sequence as SEQ ID NO:2, but which are degenerate to SEQ ID NO:2. By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid. It is well known in the art that the following codons can be used interchangeably to code for each specific amino acid:

	Phenylalanine (Phe or F)	UUU or UUC
10	Leucine (Leu or L)	UUA or UUG or CUU or CUC or CUA or CUG
	Isoleucine (Ile or I)	AUU or AUC or AUA
	Methionine (Met or M)	AUG
	Valine (Val or V)	GUU or GUC or GUA or GUG
	Serine (Ser or S)	UCU or UCC or UCA or UCG or AGU or AGC
15	Proline (Pro or P)	CCU or CCC or CCA or CCG
	Threonine (Thr or T)	ACU or ACC or ACA or ACG
	Alanine (Ala or A)	GCU or GCG or GCA or GCG
	Tyrosine (Tyr or Y)	UAU or UAC
	Histidine (His or H)	CAU or CAC
20	Glutamine (Gln or Q)	CAA or CAG
	Asparagine (Asn or N)	AAU or AAC
	Lysine (Lys or K)	AAA or AAG
	Aspartic Acid (Asp or D)	GAU or GAC
	Glutamic Acid (Glu or E)	GAA or GAG
25	Cysteine (Cys or C)	UGU or UGC
	Arginine (Arg or R)	CGU or CGC or CGA or CGG or AGA or AGG
	Glycine (Gly or G)	GGU or GGC or GGA or GGG
	Tryptophan (Trp or W)	UGG

Termination codon UAA (ochre) or UAG (amber) or UGA (opal)

It should be understood that the codons specified above are for RNA sequences. The corresponding codons for DNA have a T substituted for U.

- 5 Mutations can be made, e.g. in SEQ ID NO:1, or any of the nucleic acids set forth herein, such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the
- 10 codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less
- 15 change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein. The present invention should be considered to include sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting protein.
- 20 The following is one example of various groupings of amino acids:

Amino acids with nonpolar R groups

Alanine

Valine

Leucine

25 Isoleucine

Proline

Phenylalanine

Tryptophan

Methionine

Amino acids with uncharged polar R groups

5 Glycine

Serine

Threonine

Cysteine

Tyrosine

10 Asparagine

Glutamine

Amino acids with charged polar R groups (negatively charged at pH 6.0)

Aspartic acid

Glutamic acid

15 Basic amino acids (positively charged at pH 6.0)

Lysine

Arginine

Histidine (at pH 6.0)

Another grouping may be those amino acids with phenyl groups:

20 Phenylalanine

Tryptophan

Tyrosine

Another grouping may be according to molecular weight (i.e., size of R groups):

	Glycine	75
	Alanine	89
	Serine	105
5	Proline	115
	Valine	117
	Threonine	119
	Cysteine	121
	Leucine	131
10	Isoleucine	131
	Asparagine	132
	Aspartic acid	133
	Glutamine	146
	Lysine	146
15	Glutamic acid	147
	Methionine	149
	Histidine (at pH 6.0)	155
	Phenylalanine	165
	Arginine	174
20	Tyrosine	181
	Tryptophan	204

Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- 25 - Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH_2 can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced into a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β -turns in the protein's structure.

Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

10 A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding
15 sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

20 An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies. the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567.

An "antibody combining site" is that structural portion of an antibody molecule
25 comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

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000000" 8724960

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v), which portions are preferred for use in the therapeutic methods described herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

25 A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate

start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined T_m with washes of higher stringency, if desired.

In its primary aspect, the present invention concerns the identification of a class of DNA Polymerase III-type enzymes or complexes found in thermophilic bacteria such as *Thermus thermophilus* (*T.th.*), and other eubacteria such as *Thermatoga*, which exhibit the following characteristics, among their properties: the ability to extend a primer over a long stretch of ssDNA at elevated temperature, stimulation by its cognate sliding clamp of the type that is assembled on DNA by a clamp loader (e.g. γ complex), accessory subunits that exhibit DNA-stimulated ATPase activity at elevated temperature and/or ionic strength, and an associated 3'-5' exonuclease activity. In a particular aspect, the invention extends to Polymerase III-type enzymes derived from a broad class of thermophilic bacteria that include

polymerases isolated from the thermophilic bacteria *Thermus thermophilus* (*T.th.* polymerase), *Thermococcus litoralis* (*Tli* or VENT™ polymerase), *Pyrococcus furiosus* (*Pfu* or DEEPVENT polymerase), *Pyrococcus woosii* (*Pwo* polymerase) and other *Pyrococcus* species, *Bacillus sterothermophilus* (*Bst* polymerase), *sulfolobus acidocaldarius* (*Sac* polymerase), *thermoplasma acidophilum* (*Tac* polymerase), *Thermus favus* (*Tfl/Tub* polymerase), *Thermus ruber* (*Tru* polymerase), *Thermus brockianus* (DYNAZYME™ polymerase), *Thermotoga neapolitana* (*Tne* polymerase; See WO 96/10640), *Thermotoga maritima* (*Tma* polymerase; See U.S. Patent No. 5,374,553) and other species of the *Thermotoga* genus (*Tsp* polymerase) and

10 *Methanobacterium thermoautotrophicum* (*Mth* polymerase). The particular polymerase discussed herein by way of illustration and not limitation, is the enzyme derived from *T.th.*.

Polymerase III-type enzymes covered by the invention include those that may be prepared by purification from cellular material, as described in detail in Example 9

15 herein, as well as enzyme assemblies or complexes that comprise the combination of individually prepared enzyme subunits or components. Accordingly, the entire enzyme may be prepared by purification from cellular material, or may be constructed by the preparation of the individual components and their assembly into the functional enzyme. A representative and non-limitative protocol for the preparation of an

20 enzyme by this latter route is set forth in U.S. Patent No. 5,583,026, issued December 10, 1996, to one of the inventors herein, and the disclosure thereof is incorporated herein in its entirety for such purpose.

Likewise, individual subunits may be modified, *e.g.* as by incorporation therein of single residue substitutions to create active sites therein, for the purpose of imparting

25 new or enhanced properties to enzymes containing the modified subunits. See, for example, Tabor, S. et al. (1995) *Proc. Natl. Acad. Sci. USA*, **92(14)**:6339-6343, the disclosure of which is also incorporated herein in its entirety. Likewise, individual subunits prepared in accordance with the invention, may be used individually and for

example, may be substituted for their counterparts in other enzymes, to improve or particularize the properties of the resultant modified enzyme. Such modifications are within the skill of the art and are considered to be included within the scope of the present invention.

5

Accordingly, the invention includes the various subunits that may comprise the enzymes, and accordingly extends to the genes and corresponding proteins that may be encoded thereby, such as the α , β , γ , ϵ , τ , δ and δ' subunits, respectively. More particularly, the α subunit corresponds to *dnaE*, the β subunit corresponds to *dnaN*,
10 the ϵ subunit corresponds to *dnaQ*, and the γ and τ subunits correspond to *dnaX*.

Accordingly, the Polymerase III-type enzyme of the present invention comprises at least one gene encoding a sub unit thereof, which gene is selected from the group consisting of *dua X*, *dua Q*, *dua E* and *dua N*, and combinations thereof. More particularly, the invention extends to the nucleic acid molecule encoding them and t
15 subunits, and includes the *dua X* gene which has a nucleotide sequence as set forth in SEQ ID NO. 3, as well as conserved variants, active fragments and analogs thereof. Likewise, the nucleotide sequences encoding the α sub unit (*dna ϵ* gene). The ϵ sub unit (*dnaQ* gene) and the β sub unit (*dna N* gene) each comprise the nucleotide
20 conserved variants, active fragments and analogs thereof.

A particular Polymerase III-type enzyme in accordance with the invention may include at least one of the following sub-units:

- A. a γ subunit having an amino acid sequence selected from the formula set forth in SEQ ID NOS:4 and 5;
- 25 B. a τ subunit having an amino acid sequence corresponding to the formula set forth in SEQ ID NO:2;
- C. a ϵ subunit having an amino acid sequence corresponding to the formula set forth in SEQ ID NO:95;

D. a α subunit including an amino acid sequence corresponding to the formula set forth in SEQ ID NO:87;

E. a β subunit having an amino acid sequence corresponding to the formula set forth in SEQ ID NO:107; and

5 F. combinations of the above.

The invention also includes and extends to the use and application of the enzyme and/or one or more of its components for DNA molecule amplification and sequencing by the methods set forth hereinabove, and in greater detail later on herein.

10 One of the subunits of the invention is the γ/τ subunit encoded by a *dnaX* gene, which frameshifts as much as -2 with high efficiency, and that, upon frameshifting, leads to the addition of more than one extra amino acid residue to the C-terminus (to form the γ subunit). Further, the invention likewise extends to a *dnaX* gene derived from a thermophile such as *T.th.*, that possesses the frameshift defined herein and that codes
15 for expression of the γ and τ subunits of DNA Polymerase III.

The present invention provides methods for amplifying or sequencing a nucleic acid molecule comprising contacting the nucleic acid molecule with a composition comprising a DNA polymerase III enzyme (DNA pol III) complex, preferably a DNA pol III complex that is substantially reduced in 3'-5' exonuclease activity. DNA pol III
20 complexes used in the methods of the present invention are thermostable.

The invention also provides DNA molecules amplified by the present methods, methods of preparing a recombinant vector comprising inserting a DNA molecule amplified by the present methods into a vector, which is preferably an expression vector, and recombinant vectors prepared by these methods.

The invention also provides methods of preparing a recombinant host cell comprising inserting a DNA molecule amplified by the present methods into a host cell, which preferably a bacterial cell, most preferably an *Escherichia coli* cell; a yeast cell; or an animal cell, most preferably an insect cell, a nematode cell or a mammalian cell. The
5 invention also provides and recombinant host cells prepared by these methods.

In additional preferred embodiments, the present invention provides kits for amplifying or sequencing a nucleic acid molecule. DNA amplification kits according to the invention comprise a carrier means having in close confinement therein two or more container means, wherein a first container means contains a DNA polymerase III
10 enzyme complex and a second container means contains a deoxynucleoside triphosphate. DNA sequencing kits according to the present invention comprise a multi-protein Pol III-type enzyme complex and a second container means contains a dideoxynucleoside triphosphate. The DNA pol III contained in the container means of such kits is preferably substantially reduced in 5'-3' exonuclease activity, may be
15 thermostable, and may be isolated from the thermophilic cellular sources described above. Most preferably, the DNA pol III contained in the container means of such kits is a DNA polymerase III-type complex of a thermophile which lacks the ϵ subunit.

DNA pol III-type enzyme complexes for use in the present invention may be isolated
20 from any organism that produced the DNA pol III-type enzyme complexes naturally or recombinantly. Such enzyme complexes may be thermostable, isolated from a variety of thermophilic organisms.

The thermostable DNA polymerase III-type enzymes or complexes that are an important aspect of this invention, may be isolated from a variety of thermophilic
25 bacteria that are available commercially (for example, from American Type Culture Collection, Rockville, Maryland). Suitable for use as sources of thermostable enzymes are the thermophilic bacteria *Thermus aquaticus*, *Thermus thermophilus*,

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Thermococcus litoralis, *Pyrococcus furiosus*, *Pyrococcus woosii* and other species of the *Pyrococcus* genus, *Bacillus stearothermophilus*, *Sulfolobus acidocaldarius*, *Thermoplasma acidophilum*, *Thermus flavus*, *Thermus ruber*, *Thermus brockianus*, *Thermotoga neapolitana*, *Thermotoga maritima* and other species of the *Thermotoga* genus, and *Methanobacterium thermoautotrophicum*, and mutants of each of these species. It will be understood by one of ordinary skill in the art, however, that any thermophilic microorganism might be used as a source of thermostable DNA pol III-type enzymes and polypeptides for use in the methods of the present invention.

Bacterial cells may be grown according to standard microbiological techniques, using culture media and incubation conditions suitable for growing active cultures of the particular thermophilic species that are well-known to one of ordinary skill in the art (see, e.g., Brock, T.D., and Freeze, H., *J. Bacteriol.* 98(1):289-297 (1969); Oshima, T., and Imahori, K. *Int. J. Syst. Bacteriol.* 24(1):102-112(1974)). Thermostable DNA pol III complexes may then be isolated from such thermophilic cellular sources as described for thermolabile complexes above.

As stated above and in accordance with the present invention, nucleic acid molecules may be amplified according to any of the literature-described manual or automated amplification methods. Such methods includes, but are not limited to, PCR (U.S. Patent Nos. 4,683,195 and 4,683,202), Strand Displacement Amplification (SDA; U.S. Patent No. 5,455,166; EP 0 684 315), and Nucleic Acid Sequence-Based Amplification (NASBA; U.S. Patent No. 5,409,818; EP 0 329 822). Most preferably, nucleic acid molecules are amplified by the methods of the present invention using PCR-based amplification techniques.

In the initial steps of each of these amplification methods, the nucleic acid molecule to be amplified is contacted with a composition comprising a DNA polymerase belonging to the evolutionary "family A" class (e.g. *Taq* DNA pol I or *E. coli* pol I) or the "family B" class (e.g. Vent and *Pfu* DNA polymerases -- see Ito, J., and Braithwaite, D., *Nucl. Acids Res.* 19(15):4045-4057 (1991)). All of these DNA

polymerases are present as single subunits and are primarily involved in DNA repair. In contrast, the DNA pol III-type enzymes are multisubunit complexes that mainly function in the replication of the chromosome, and the subunit containing the DNA polymerase activity is in the "family C" class.

- 5 Thus, in amplifying a nucleic acid molecule according to the methods of the present invention, the nucleic acid molecule is contacted with a composition comprising a thermostable DNA pol III-type enzyme complex. The DNA pol III-type complexes used in the present methods are preferably substantially reduced in 3'-5' exonuclease activity (*i.e.*, they are "exo-").
- 10 Once the nucleic acid molecule to be amplified is contacted with the DNA pol III-type complex, the amplification reaction may proceed according to standard protocols for each of the above-described techniques. Since most of these techniques comprise a high-temperature denaturation step, if a thermolabile DNA pol III-type enzyme complex (such as *E. coli* DNA pol III *exo*-) is used in nucleic acid amplification by
- 15 any of these techniques the enzyme would need to be added at the start of each amplification cycle, since it would be heat-inactivated at the denaturation step. However, a thermostable DNA pol III-type complex used in these methods need only be added once at the start of the amplification (as for *Taq* DNA polymerase in traditional PCR amplifications), as its activity will be unaffected by the high
- 20 temperature of the denaturation step. It should be noted, however, that because DNA pol III-type enzymes have a much more rapid rate of nucleotide incorporation than the polymerases commonly used in these amplification techniques, the cycle times may need to be adjusted to shorter intervals than would be standard.

- In an alternative preferred embodiment, the invention provides methods of extending
- 25 primers for several kilobases, a reaction that is central to amplifying large nucleic acid molecules, by a technique commonly referred to as "long PCR" (Barnes, W.M., *Proc.*

Natl. Acad. Sci. USA 91:2216-2220 (1994); Cheng. S. *et al.*, *Proc. Natl. Acad. Sci. USA* 91:5659-5699 (1994)).

In such a method the target primed DNA can contain a single strand stretch of DNA to be copied into the double strand form of several or tens of kilobases. The reaction is performed in a suitable buffer, preferably Tris, at a pH of between 5.5 - 9.5, preferably 7.5. The reaction also contains $MgCl_2$ in the range 1 mM to 10 mM, preferably 8 mM, and may contain a suitable salt such as NaCl, KCl or sodium or potassium acetate. The reaction also contains ATP in the range of 20 μ M to 1 mM, preferably 0.5 mM, that is needed for the clamp loader to assemble the clamp onto the primed template, and a sufficient concentration of deoxynucleoside triphosphates in the range of 50 μ M to 0.5 mM, preferably 60 μ M for chain extension. The reaction contains a sliding clamp, such as the β subunit, in the range of 20ng to 200 ng, preferably 100 ng, for action as a clamp to stimulate the DNA polymerase. The chain extension reaction contains a DNA polymerase and a clamp loader, that could be added either separately or as a single Pol III* -like particle, preferably as a Pol III* like particle that contains the DNA polymerase and clamp loading activities. The Pol III-type enzyme is added preferably at a concentrations of about 0.0002-200 units per milliliter, about 0.002-100 units per milliliter, about 0.2-50 units per milliliter, and most preferably about 2-50 units per milliliter. The reaction is incubated at elevated temperature, preferably 60°C or more, and could include other proteins to enhance activity such as a single strand DNA binding protein.

In another preferred embodiment, the invention provides methods of extending primers on linear templates in the absence of the clamp loader. In this reaction, the primers are annealed to the linear DNA, preferably at the ends such as in standard PCR applications. The reaction is performed in a suitable buffer, preferably Tris, at a pH of between 5.5 - 9.5, preferably 7.5. The reaction also contains $MgCl_2$ in the range of 1 mM to 10 mM, preferably 8 mM, and may contain a suitable salt such as NaCl, KCl or sodium or potassium acetate. The reaction also contains a sufficient

concentration of deoxynucleoside triphosphates in the range of 50 μ M to 0.5 mM, preferably 60 μ M for chain extension. The reaction contains a sliding clamp, such as the β subunit, in the range of 20ng to 20 μ g, preferably 7 μ g, for ability to slide on the end of the DNA and associate with the polymerase for action as a clamp to stimulate

5 the DNA polymerase. The chain extension reaction also contains a Pol III-type polymerase subunit such as α , core, or a Pol III* -like particle. The Pol III-type enzyme is added preferably at a concentrations of about 0.0002-200 units per milliliter, about 0.002-100 units per milliliter, about 0.2-50 units per milliliter, and most preferably about 2-50 units per milliliter. The reaction is incubated at elevated

10 temperature, preferably 60°C or more, and could include other proteins to enhance activity such as a single strand DNA binding protein.

The methods of the present invention thus will provide high-fidelity amplified copies of a nucleic acid molecule in a more rapid fashion than traditional amplification methods using the repair-type enzymes.

- 15 These amplified nucleic acid molecules may then be manipulated according to standard recombinant DNA techniques. For example, a nucleic acid molecule amplified according to the present methods may be inserted into a vector, which is preferably an expression vector, to produce a recombinant vector comprising the amplified nucleic acid molecule. This vector may then be inserted into a host cell,
- 20 where it may, for example, direct the host cell to produce a recombinant polypeptide encoded by the amplified nucleic acid molecule. Methods for inserting nucleic acid molecules into vectors, and inserting these vectors into host cells, are well-known to one of ordinary skill in the art (see, *e.g.*, Maniatis, T., *et al.*, *Molecular Cloning, A Laboratory Manual*. Boca Raton, Florida: CRC Press (1992)).
- 25 Alternatively, the amplified nucleic acid molecules may be directly inserted into a host cell, where it may be incorporated into the host cell genome or may exist as an extrachromosomal nucleic acid molecule, thereby producing a recombinant host cell.

Methods for introduction of a nucleic acid molecule into a host cell, including calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods, are described in many standard laboratory manuals (see *e.g.*, Davis et al., *Basic Methods In Molecular*
 5 *Biology* (1986)).

For each of the above techniques wherein an amplified nucleic acid molecule is introduced into a host cell via a vector or via direct introduction, preferred host cells include but are not limited to a bacterial cell, a yeast cell, or an animal cell. Bacterial host cells preferred in the present invention are *E. coli*, *Bacillus* spp., *Streptomyces*
 10 spp., *Erwinia* spp., *Klebsiella* spp. and *Salmonella typhimurium*. Preferred as a host cell is *E. coli*, and particularly preferred are *E. coli* strains DH10B and Stbl2, which are available commercially (Life Technologies, Inc. Gaithersburg, Maryland). Preferred animal host cells are insect cells, nematode cells and mammalian cells. Insect host cells preferred in the present invention are *Drosophila* spp. cells,
 15 *Spodoptera* Sf9 and Sf21 cells, and *Trichoplusia* High-Five cells, each of which is available commercially (*e.g.*, from Invitrogen; San Diego, California). Preferred nematode host cells are those derived from *C. elegans*, and preferred mammalian host cells are those derived from rodents, particularly rats, mice or hamsters, and primates, particularly monkeys and humans. Particularly preferred as mammalian host cells are
 20 CHO cells, COS cells and VERO cells.

By the present invention, nucleic acid molecules may be sequenced according to any of the literature-described manual or automated sequencing methods. Such methods include, but are not limited to, dideoxy sequencing methods ("Sanger sequencing"; Sanger, F., and Coulson, A.R., *J. Mol. Biol.* 94:444-448 (1975); Sanger, F., *et al.*,
 25 *Proc. Natl. Acad. Sci. USA* 74:5463-5467 (1977); U.S. Patent Nos. 4,962,022 and 5,498,523), as well as more complex PCR-based nucleic acid fingerprinting techniques such as Random Amplified Polymorphic DNA (RAPD) analysis (Williams, J.G.K. *et al.*, *Nucl. Acids Res.* 18(22):6531-6535, 1990). Arbitrarily

Primed PCR (AP-PCR; Welsh, J., and McClelland. M., *Nucl. Acids Res.* 18(24):7213-7218, 1990), DNA Amplification Fingerprinting (DAF; Caetano-Anollés et al., *Bio/Technology* 9:553-557, 1991), microsatellite PCR or Directed Amplification of Minisatellite-region DNA (DAMD; Heath, D.D., *et al.*, *Nucl. Acids Res.* 21(24):5782-5785, 1993). and Amplification Fragment Length Polymorphism (AFLP) analysis (EP 0 534 858; Vos. P., *et al.*, *Nucl. Acids Res.* 23(21):4407-4414, 1995; Lin, J.J., and Kuo, J., *FOCUS* 17(2):66-70, 1995).

As described above for amplification methods, the nucleic acid molecule to be sequenced by these methods is typically contacted with a composition comprising a type A or type B DNA polymerase. By contrast, in sequencing a nucleic acid molecule according to the methods of the present invention, the nucleic acid molecule is contacted with a composition comprising a thermostable DNA pol III-type enzyme complex instead of necessarily using a DNA polymerase of the family A or B classes. As for amplification methods, the DNA pol III-type complexes used in the nucleic acid sequencing methods of the present invention are preferably substantially reduced in 5'-3' exonuclease activity; most preferable for use in the present methods is a DNA polymerase III-type complex which lacks the ϵ subunit. DNA pol III-type complexes used for nucleic acid sequencing according to the present methods are used at the same preferred concentration ranges described above for long chain extension of primers.

Once the nucleic acid molecule to be sequenced is contacted with the DNA pol III complex, the sequencing reactions may proceed according to the protocols disclosed in the above-referenced techniques.

As discussed above, the invention extends to kits for use in nucleic acid amplification or sequencing utilizing DNA polymerase III-type enzymes according to the present methods. A DNA amplification kit according to the present invention may comprise a carrier means, such as vials, tubes, bottles and the like. A first such container means

may contain a DNA polymerase III-type enzyme complex, and a second such container means may contain a deoxynucleoside triphosphate. The amplification kit encompassed by this aspect of the present invention may further comprise additional reagents and compounds necessary for carrying out standard nucleic amplification
5 protocols (See U.S. Patent Nos. 4,683,195 and 4,683,202, which are directed to methods of DNA amplification by PCR).

Similarly, a DNA sequencing kit according to the present invention comprises a carrier means having in close confinement therein two or more container means, such as vials, tubes, bottles and the like. A first such container means may contain a DNA
10 polymerase III-type enzyme complex, and a second such container means may contain a dideoxynucleoside triphosphate. The sequencing kit may further comprise additional reagents and compounds necessary for carrying out standard nucleic sequencing protocols, such as pyrophosphatase, agarose or polyacrylamide media for formulating sequencing gels, and other components necessary for detection of
15 sequenced nucleic acids (See U.S. Patent Nos. 4,962,020 and 5,498,523, which are directed to methods of DNA sequencing).

The DNA polymerase III-type complex contained in the first container means of the amplification and sequencing kits provided by the invention is preferably a thermostable DNA polymerase III-type enzyme complex and more preferably a DNA
20 polymerase III-type enzyme complex that is substantially reduced in 3-5' exonuclease activity. Naturally, the foregoing methods and kits are presented as illustrative and not restrictive of the use and application of the enzymes of the invention for DNA molecule amplification and sequencing. Likewise, the applications of specific embodiments of the enzymes, including conserved variants and active fragments
25 thereof are considered to be disclosed and included within the scope of the invention.

As discussed earlier, individual subunits could be modified to customize enzyme construction and corresponding use and activity. For example, the region of α that

interacts with β could be subcloned onto another DNA polymerase, thereby causing β to enhance the activity of the recombinant polymerase. Alternatively, the β clamp could be modified to function with another protein or enzyme thereby enhancing its activity or acting to localize its action to a particular targeted DNA. Finally, the polymerase active site could be modified to enhance its action, for example changing Tyrosine enabling more equal site stoppage with the four ddNTPs (Tabor et al. 1995). This represents a particular non-limiting illustration of the scope and practice of the present invention with reference to the utility of individual subunits hereof.

Accordingly and as stated above, the present invention also relates to a recombinant DNA molecule or cloned gene, or a degenerate variant thereof, which encodes any one or all of the subunits of the DNA Polymerase III-type enzymes of the present invention, or active fragments thereof. In the instance of the τ subunit, a predicted molecular weight of about 58 kD and an amino acid sequence set forth in SEQ ID NOS:4 or 5 is comprehended; preferably a nucleic acid molecule, in particular a recombinant DNA molecule or cloned gene, encoding the 58 kD subunit of the Polymerase III of the invention, that has a nucleotide sequence or is complementary to a DNA sequence shown in FIGURES 4A and 4B (SEQ ID NO:1), and the coding region for *dnaX* set forth in FIGURE 4C (SEQ ID NO:3). The γ subunit is smaller, and is approximately 50 kD, depending upon the extent of the frameshift that occurs. More particularly, and as set forth in FIGURE 4E (SEQ ID NO:4), the γ subunit defined by a -1 frameshift possesses a molecular weight of 50.8 kD, while the γ subunit defined by a -2 frameshift, set forth in FIGURE 4F (SEQ ID NO:5), possesses a molecular weight of 49.8 kD.

As discussed above, the invention also extends to the genes including *dnaX*, *dnaQ*, *dnaE* and *dnaN*, that have been isolated and purified from *Thermus thermophilus*, to corresponding vectors for the genes, and particularly, to the vectors pET*dnaX* and pET*dnaN*, and to host cells including such vectors. In this connection, probes have been prepared which hybridize to the DNA polymerase III-type enzymes of the

present invention, and which are selected from the group consisting of the oligonucleotide defined in SEQ ID NO:6; the oligonucleotide defined in SEQ ID NO:8; the oligonucleotide defined in SEQ ID NO:10; the oligonucleotide defined in SEQ ID NO:11; the oligonucleotide defined in SEQ ID NO:12; the oligonucleotide defined in SEQ ID NO:13; the oligonucleotide defined in SEQ ID NO:14; the oligonucleotide defined in SEQ ID NO:15, and the oligonucleotide defined in SEQ ID NO:16.

The methods of the invention include a method for producing a recombinant thermostable DNA polymerase III-type enzyme from a thermophilic bacterium such as *Thermus thermophilus* which comprises culturing a host cell transformed with a vector of the invention under conditions suitable for the expression of the present DNA polymerase III. Another method includes a method for isolating a target DNA fragment consisting essentially of a DNA coding for a thermostable DNA polymerase III-type enzyme from a thermophilic bacterium comprising the steps of:

- (a) forming a genomic library from the bacterium;
- (b) transforming or transfecting an appropriate host cell with the library of step (a);
- (c) contacting DNA from the transformed or transfected host cell with a DNA probe which hybridizes to a DNA fragment selected from the group consisting of the DNA fragments defined in SEQ ID NO:6 and the DNA fragments defined in SEQ ID NO:8 or the oligonucleotides set forth above; wherein hybridization is conducted under the following conditions:
 - i) hybridization: 1% crystalline BSA (fraction V) (Sigma), 1 mM EDTA, 0.5 M NaHPO₄ (pH 7.2), 7% SDS at 65°C for 12 hours and;
 - ii) wash: 5 x 20 minutes with wash buffer consisting of 0.5% BSA, fraction V), 1mM Na₂EDTA, 40 mM NaHPO₄ (pH 7.2), and 5% SDS;
 - (d) assaying the transformed or transfected cell of step (c) which hybridizes to the DNA probe for DNA polymerase III-type activity; and

(e) isolating a target DNA fragment which codes for the thermostable DNA polymerase III-type enzyme.

Also, antibodies including both polyclonal and monoclonal antibodies, and the DNA Polymerase III-like enzyme complex and/or their γ and τ subunits or α subunit may
 5 be used in the preparation of the enzymes of the present invention as well as other enzymes of similar thermophilic origin. For example, the DNA Polymerase III-type complex or its subunits may be used to produce both polyclonal and monoclonal antibodies to themselves in a variety of cellular media, by known techniques such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and
 10 myeloma cells.

The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al.,
 15 "Hybridoma Techniques" (1980); Hammerling et al., "Monoclonal Antibodies And T-cell Hybridomas" (1981); Kennett et al., "Monoclonal Antibodies" (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,451,570; 4,466,917; 4,472,500; 4,491,632; 4,493,890.

Methods for producing polyclonal anti-polypeptide antibodies are well-known in the
 20 art. See U.S. Patent No. 4,493,795 to Nestor et al. A monoclonal antibody, typically containing Fab and/or F(ab')₂ portions of useful antibody molecules, can be prepared using the hybridoma technology described in *Antibodies - A Laboratory Manual*, Harlow and Lane, eds., Cold Spring Harbor Laboratory, New York (1988), which is incorporated herein by reference. Briefly, to form the hybridoma from which the
 25 monoclonal antibody composition is produced, a myeloma or other self-perpetuating cell line is fused with lymphocytes obtained from the spleen of a mammal hyperimmunized with an elastin-binding portion thereof.

A monoclonal antibody useful in practicing the present invention can be produced by initiating a monoclonal hybridoma culture comprising a nutrient medium containing a hybridoma that secretes antibody molecules of the appropriate antigen specificity. The culture is maintained under conditions and for a time period sufficient for the
5 hybridoma to secrete the antibody molecules into the medium. The antibody-containing medium is then collected. The antibody molecules can then be further isolated by well-known techniques.

Media useful for the preparation of these compositions are both well-known in the art and commercially available and include synthetic culture media, inbred mice and the
10 like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco et al., *Virol.* 8:396 (1959)) supplemented with 4.5 gm/l glucose, 20 mm glutamine, and 20% fetal calf serum. An exemplary inbred mouse strain is the Balb/c.

Another feature of this invention is the expression of the DNA sequences disclosed
15 herein. As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host.

Such operative linking of a DNA sequence of this invention to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision
20 of an initiation codon, ATG, in the correct reading frame upstream of the DNA sequence.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic
25 DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col El, pCR1, pBR322, pMB9 and their derivatives,

Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the *lac* system, the *trp* system, the *TAC* system, the *TRC* system, the *LTR* system, the major operator and promoter regions of phage λ , the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the promoters of the yeast α -mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, such as CHO, R1.1, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue culture.

It will be understood that not all vectors, expression control sequences and hosts will
25 function equally well to express the DNA sequences of this invention. Neither will all
hosts function equally well with the same expression system. However, one skilled in
the art will be able to select the proper vectors, expression control sequences, and

hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector, the host must be considered because the vector must function in it. The vector's copy number, the ability to control that copy number, and the expression of any other

5 proteins encoded by the vector, such as antibiotic markers, will also be considered.

In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly with regard to potential secondary structures. Suitable

10 unicellular hosts will be selected by consideration of, e.g., their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products.

15 Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences of this invention on fermentation or in large scale animal culture.

It is further intended that analogs may be prepared from nucleotide sequences of the protein complex/subunit derived within the scope of the present invention. Analogs,

20 such as fragments, may be produced, for example, by pepsin digestion of bacterial material. Other analogs, such as muteins, can be produced by standard site-directed mutagenesis of *dnaX*, *dnaE*, *dnaQ* or *dnaN* coding sequences. Especially useful may be a mutation in *dnaE* that provides the polymerase with the ability to incorporate all four ddNTPs with equal efficiency thereby producing an even binding pattern in

25 sequencing gels, as discussed above and with reference to Tabor et al. 1995, *supra*.

- As mentioned above, a DNA sequence corresponding to *dnaX*, *dnaQ*, *dnaE* or *dnaN*, or encoding the subunits of the DNA Polymerase III of the invention can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the amino acid sequence of the subunit(s) of interest. In
- 5 general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, *Nature*, **292**:756 (1981); Nambair et al., *Science*, **223**:1299 (1984); Jay et al., *J. Biol. Chem.*, **259**:6311 (1984).
- 10 Synthetic DNA sequences allow convenient construction of genes which will express DNA Polymerase III analogs or "muteins". Alternatively, DNA encoding muteins can be made by site-directed mutagenesis of native *dnaX*, *dnaQ*, *dnaE* or *dnaN* genes or their corresponding cDNAs, and muteins can be made directly using conventional polypeptide synthesis.
- 15 A general method for site-specific incorporation of unnatural amino acids into proteins is described in Christopher J. Noren, Spencer J. Anthony-Cahill, Michael C. Griffith, Peter G. Schultz, *Science*, **244**:182-188 (April 1989). This method may be used to create analogs with unnatural amino acids.

GENERAL DESCRIPTION

- 20 As discussed above, the present invention has as one of its characterizing features, that a Polymerase III-type enzyme as defined hereinabove, has been discovered in a thermophile, that has the structure and function of a chromosomal replicase. This structure and function confers significant benefit when the enzyme is employed in procedures such as PCR where speed and accuracy of DNA reconstruction is crucial.

Chromosomal replicases are composed of several subunits in all organisms (Kornberg and Baker, 1992). In keeping with the need to replicate long chromosomes, replicases are rapid and highly processive multiprotein machines. All cellular replicases examined to date derive their processivity from one subunit that is shaped like a ring and completely encircles DNA (Kuriyan and O'Donnell, 1993; Kelman and O'Donnell, 1994). This "sliding clamp" subunit acts as a mobile tether for the polymerase machine (Stukenberg et. al., 1991). The sliding clamp does not assemble onto the DNA by itself, but requires a complex of several proteins, called a "clamp loader" which couples ATP hydrolysis to the assembly of sliding clamps onto DNA (O'Donnell et. al., 1992). Hence, Pol III-type cellular replicases are comprised of three components: a clamp, a clamp loader, and the DNA polymerase.

An overall goal is to identify and isolate all of the genes encoding the replicase subunits from a thermophile for expression and purification in large quantity. Following this, the replication apparatus can be reassembled from individual subunit components for use in kits, PCR, sequencing and diagnostic applications (Onrust et. al., 1995).

As a beginning to identify and characterize the replicase of a thermophile, we started by looking for a homologue to the prokaryotic *dnaX* gene which encode subunits (γ and τ) of the replicase. The *dnaX* gene has another homologue, *holB*, which encodes yet another subunit (δ') of the replicase. The amino acid sequence of δ' (encoded by *holA*) and τ/γ subunits (encoded by *dnaX*) are particularly highly conserved in evolution from prokaryotes to eukaryotes (Chen et. al., 1992; O'Donnell et. al., 1993; Onrust et. al., 1993; Carter et. al., 1993; Cullman et. al., 1995).

The organism chosen for study and exposition herein is the exemplary extreme thermophile, *Thermus thermophilus* (T.th.). It is understood that other members of the class such as the eubacterium *Thermatoga* are expected to be analogous in both structure and function. Thus, the investigation of *T.th.* proceeded and initially, a T.th.

homologue of *dnaX* was identified. The gene encodes a full length protein of 529 amino acids. The amino terminal third of the sequence shares over 50% homology to *dnaX* genes as divergent as *E. coli* (gram negative) and *B. subtilis* (gram positive).

The *T.th.* *dnaX* gene contains a DNA sequence that provides a translational frameshift signal for production of two proteins from the same gene. Such frameshifting has been documented only in the case of *E. coli* (Tsuchihashi and Kornberg, 1990; Flower and McHenry, 1990; Blinkowa and Walker, 1990). No frameshifting has been documented to occur in the *dnaX* homologues (RFC subunit genes) of yeast and humans (Eukaryotic kingdom).

- 10 The presence of a *dnaX* gene that produces two subunits implies that *T.th.* has a clamp loader (γ) and is organized by τ into a three component Pol III-type replicase. The three components of its replicase may be organized into a holoenzyme particle like the replicative DNA polymerase of *Escherichia coli*, DNA polymerase III holoenzyme. The *E. coli* DNA polymerase III holoenzyme contains 10 different subunits, some in
- 15 copies of two or more for a total composition of 18 polypeptide chains (Baker and Kornberg, 1992; Onrust et. al., 1995). The holoenzyme is composed of three major activities: the 3-subunit DNA polymerase core ($\alpha\epsilon\theta$), the β subunit DNA sliding clamp, and the 5-subunit γ complex clamp loader ($\gamma\delta\delta'\chi\psi$). This 3 component strategy generalizes to eukaryotes which utilize a clamp (PCNA) and a 5-subunit RFC
- 20 clamp loader (RFC) which provide processivity to DNA polymerase δ (reviewed in Kelman and O'Donnell, 1994).

In *E. coli*, the three components are organized into one holoenzyme particle by the τ subunit, that acts as a "glue" protein (Onrust and O'Donnell, 1995). One dimer of τ holds together two core polymerases into one particle which are utilized for the

25 coordinated and simultaneous replication of both strands of duplex DNA (McHenry, 1982; Maki et. al., 1988; Yuzhakov et. al., 1996). The "glue" protein τ subunit also binds one clamp loader (called γ complex) thereby acting as a scaffold for a large superstructure assembly called DNA polymerase III holoenzyme. The gene encoding

- τ , called *dnaX*, also encodes the γ subunit of DNA polymerase III. The β subunit then associates with Pol III to form the DNA polymerase III holoenzyme. The γ subunit is approximately 2/3 the length of τ . γ shares the N-terminus of τ , but is truncated by a translational frameshifting mechanism that, after the shift, encounters a stop codon within two amino acids (Tsuchihashi and Kornberg, 1990; Flower and McHenry, 1990; Blinkowa and Walker, 1990). Hence, γ is the N-terminal 453 amino acids of τ , but contains one unique residue at the C-terminus (the penultimate codon encodes a Lys residue which is the same sequence as if the frameshift did not take place). This frameshift is highly efficient and occurs approximately 50% of the time.
- 10 The sequence of the γ and τ subunits encoded by the *dnaX* gene are homologous to the clamp loading subunits in all other organisms extending from gram negative bacteria through gram positive bacteria, the Archaeae Kingdom and the Eukaryotic Kingdom from yeast to humans (O'Donnell et al., 1993). All of these organisms utilize a three component replicase (DNA polymerase, clamp and clamp loader) and in
- 15 these cases the 3 components appear to behave as independent units in solution rather than forming a large holoenzyme superstructure. For example, in eukaryotes from yeast to humans, the clamp loader is the five subunit RFC, the clamp is PCNA and the polymerases δ and ϵ are all stimulated by the PCNA clamp assembled onto primed DNA by RFC (reviewed in Kelman et. al., 1994).
- 20 The discovery of a *dnaX* gene in *T.th.* provided confidence that thermophilic bacteria would contain a three component Pol III-type enzyme. Hence, we proceeded to identify the *dnaQ* and *dnaN* genes encoding, respectively, the proofreading 3'-5' exonuclease, and the β DNA sliding clamp subunits of a Pol III-type enzyme. Following this, we purified from extracts of *T.th.* cells, a Pol III-type enzyme. This
- 25 enzyme preparation had the unique property of extending a single primer around a long 7.2 kb single strand DNA genome of M13mp18 bacteriophage. Such a primer extension assay serves as a tool to detect and identify the Pol III-type of enzyme in cell extracts. The enzyme was confirmed to be a Pol III-type enzyme based on its

reactivity with antibody directed against the *E. coli* α subunit (the DNA polymerase subunit) and antibody directed against *E. coli* γ subunit. Proteins corresponding to α , τ , γ , δ and δ' were easily visible and will lend themselves to identification of the genes through use of peptide microsequencing followed by primer design for PCR
 5 amplification. From this DNA pol III-type preparation we obtained peptide sequence of the α subunit enabling us to obtain the *dnaE* gene encoding the α subunit (DNA polymerase) of the Pol III-type enzyme.

These methods should be widely applicable to other thermophilic bacteria. Additional antibody reagents against other Pol III-type enzyme components, such as RFC
 10 subunits, DNA polymerase delta, epsilon or beta, and the PCNA clamp from known organisms can be made quite easily as polyclonal or monoclonal antibody preparations using as antigen either naturally purified sequence, recombinant sequence, or synthetic peptide sequence. Examples of known sequences of these Pol III-type enzymes are to be found in: 1) DNA polymerases (Braithwaite and Ito, 1993),
 15 RFC clamp loaders (Cullman et. Al., 1995), and PCNA (Ielman and O'Donnell, 1995).

Braithwaite, D.K. and Ito, J. (1993) Compilation, alignment, and phylogenetic relationships of DNA polymerases. *Nuc. Acids Res.* 21, 787-802.

Cullman G., Fein, K., Kobayashi, R., and Stillman. B. (1995) Characterization of the
 20 five replication factor C genes of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 15, 4661-4671.

Kelman, Z., and O'Donnell, M. (1995) Structural and functional similarities of prokaryotic and eukaryotic DNA polymerase sliding clamps. *Nucl Acids Res.* 23, 3613-3620.

The remaining genes of Pol III needed for efficient extension of primed templates should be easy to obtain from the *T.th.* Pol III by similar methods as those described herein. These genes will provide the subunit preparations through use of standard recombinant techniques and protein purification protocols. The protein subunits can then be used to reconstitute the enzyme complexes as they exist in the cell. This type of reconstitution of Pol III has been demonstrated using the protein subunits of DNA polymerase III holoenzyme from *E. coli* to assemble the entire particle. See *e.g.*, U.S. Patent No. 5,583,026, issued December, 1996, O'Donnell, M.E.; and U.S. Patent No. 5,668,004, issued September, 1997, both to one of the inventors herein, and Onrust et. al. 1995b. The disclosures of these references are incorporated herein in their entireties.

The following experiments illustrate the identification and characterization of the enzymes and constructs of the present invention. Accordingly, in Examples 1-8 below, the identification and expression of the γ and τ is presented, as the first step in the elucidation of the Polymerase III reflective of the present invention. Examples 9-13 which follow set forth the protocol for the purification of the remainder of the subunits of the enzyme that represent substantial entirety of the functional replicative machinery of the enzyme.

EXAMPLE 1

20

EXPERIMENTAL PROCEDURES

Materials - DNA modification enzymes were from New England Biolabs. Labelled nucleotides were from Amersham, and unlabeled nucleotides were from New England Biolabs. The Alter-1 vector was from Promega. pET plasmids and *E. coli* strains, BL21(DE3) and BL21(DE3)pLysS, were from Novagen. Oligonucleotides were from Operon. Buffer A is 20mM Tris-HCl (pH 7.5), 0.1mM EDTA, 5mMDTT, and 10% glycerol.

Genomic DNA

Thermus thermophilus (strain HB8) was obtained from the American Type Tissue Collection. Genomic DNA was prepared from cells grown in 0.1 l of (Thermus medium N697 (ATCC: 4 γ yeast extract, 8.0 g polypeptone (BBL 11910), 2.0 g NaCl, 5 30.0 g agar, 1.0 L distilled water) at 75°C overnight. Cells were collected by centrifugation at 4°C and the cell pellet was resuspended in 25 ml of 100 mM Tris-HCl (pH 8.0), 0.05 M EDTA, 2 mg/ml lysozyme and incubated at room temperature for 10 min. Then 25 ml 0.10 M EDTA (pH 8.0), 6% SDS was added and mixed followed by 60 ml of phenol. The mixture was shaken for 40 min. followed by 10 10000 X G for 10 min. at room temperature. The upper phase (50 ml) was removed and mixed with 50 ml of phenol:chloroform (50:50 v/v) for 30 min. followed by centrifugation for 10 min. at room temperature. The upper phase was decanted and the DNA was precipitated upon addition of 1/10th volume 3 M sodium acetate (pH 6.5) and 1 volume ethanol. The precipitate was collected by 15 centrifugation and washed twice with 2 ml of 80% ethanol, dried and resuspended in 1 ml T.E. buffer (10mM Tris Hcl (pH 7.5), 1mM EDTA).

Cloning of *dnaX* - DNA oligonucleotides for amplification of *T.th.* genomic DNA were as follows. The upstream 32mer 20 (5'-CGCAAAGCTTCACGCSTACCTSTTCTCCGGSAC-3') (S indicates a mixture of G and C) consists of a Hind III site within the first 9 nucleotides (underlined) followed by codons encoding the following sequence (HAYLFSGT). The downstream 34 mer (5'-CGCGAATTCGTGCTCSGGSGGCTCCTCSAGSGTC-3') consists of an EcoRI site (underlined) followed by codons encoding the sequence 25 KTLEEPPEH on the complementary strand. The amplification reactions contained 10 ng *T.th.* genomic DNA, 0.5 mM of each primer, in a volume of 100 µl of Vent polymerase reaction mixture according to the manufacturers instructions (10 µl ThermoPol Buffer, 0.5 mM each dNTP and 0.5 mM MgSO₄). Amplification was performed using the following cycling scheme: 5 cycles of: 30 s at 95.5°C, 30 s at 30 40°C, 2 min. at 72°C; 5 cycles of: 30 s at 95.5°C, 30 s at 45°C, and 2 min. at 72°C;

and 30 cycles of: 30 s at 95.5°C, 30 s at 50°C, and 30 s at 72°C. Products were visualized in a 1.5 % native agarose gel.

Genomic DNA was digested with either XhoI, XbaI, StuI, PstI, NcoI, MluI, KpnI, HindIII, EcoRI, EagI, BglI, or BamHI, followed by Southern analysis in a native
5 agarose gel (Maniatis et. al., 1982). Approximately 0.5 µg of digest was analyzed in each lane of a 0.8 % native agarose gel followed by transfer to an MSI filter (Micron Separations Inc.). The transfer included the following steps:

1. The agarose gel was soaked in 500 ml of 1% HCl with gentle shaking for 10 min.
2. Then the gel was soaked in 500 ml of 0.5 M NaOH + 1.5 M NaCl for 40 min.
- 10 3. After that the gel was soaked in 500 ml of 1M ammonium acetate for 1 h.
4. The DNA was transferred to the MSI filter with the use of blotting paper for 4 h.
5. The filter was kept at 80°C for 15 min. in the oven.
6. The pre-hybridization step was run in 10 ml of Hybridization solution (1% crystalline BSA (fraction V) (Sigma), 1 mM EDTA, 0.5 M NaHPO₄ (pH 7.2), 7%
15 SDS) at 65°C for 30 min.
7. The probe, radiolabelled by the random priming method (see below), was added to the pre-hybridization solution and kept at 65°C for 12 h.
8. The filter was washed with low stringency with 200 ml of the wash buffer (0.5% BSA, fraction V), 1mM Na₂EDTA, 40 mM NaHPO₄ (pH 7.2), 5% SDS with gentle
20 shaking for 20 min. This step was repeated 5 times. followed by exposure to X-ray film (XAR-5, Kodak).

As a probe, the PCR product was radiolabelled by random as follows.

1. 14 ml of the mixture containing 0.2 µg of PCR product DNA, 1 µg of the pd(N6) (Promega) and 2.5 ml of the 10X Klenow reaction buffer (100 mM Tris-HCl (pH 7.5),
25 50 mM MgCl₂, 75 mM dithiothreitol) were boiled for 10 min. and then kept at 4°C .
2. The reaction volume was increased up to 25 µl, containing in addition 33 µM of each dNTP, except dATP, 10 µCi [α-³²P] dATP (800 Ci/mM), and 2 units of Klenow enzyme. The reaction mixture was incubated 1.5 h.

3. 2 mg of sonicated herring sperm DNA (GibcoBRL) was added to the reaction and the volume was increased to 2 ml using hybridization solution. The sample was then boiled for 10 min.

- A genomic library of XbaI digested DNA was prepared upon treating 1 µg genomic T.th. DNA with 10 units of XbaI in 100 µl of NEBuffer N2 (50 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT) for 2 h at 37°C. The digested DNA was purified by phenol chloroform extraction and ethanol precipitation. The Alter-1 vector (0.5 µg)(Promega) was digested with 1 unit of XbaI in NEBuffer N2 and then purified by phenol/chloroform extraction and ethanol precipitation. One microgram of genomic digest was incubated with 0.05 µg of digested Alter-1 and 20 U of T4 ligase in 30 µl of ligase buffer (50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 10 mM DTT and 1 mM ATP) at 15°C for 12 h. The ligation reaction was transformed into the DH5α strain of *E. coli* and transformants were plated on LB plates containing ampicillin and screened for the *dnaX* insert using the radiolabelled PCR probe as follows:
1. The colonies tested were lifted onto MSI filters, approximately 100 colonies to each filter.
 2. The filters, removed from the LB/Tc plates, were placed side up on a sheet of Whatman 3 MM paper soaked with 0.5 M NaOH for 5 min.
 3. The filters were transferred to a sheet of paper soaked with 1 M Tris-HCl (pH 7.5) for 5 min.
 4. The filters were placed on a sheet of paper soaked in 0.5 M Tris-HCl (pH 7.5), 1.25 M NaCl for 5 min.
 5. After drying by air, the filters were heated in the oven 80°C for 15 min. and then were analyzed by Southern hybridization.

Plasmid DNA was prepared from 20 positive colonies; of these 6 contained the expected 4 kb insert when digested with XbaI. Sequencing of the insert was

performed by the Sanger method using the Vent polymerase sequencing kit according to the manufacturers instructions (New England Biolabs).

Identification of the *dnaX* gene

The *dnaX* genes of the gram negative, *E. coli*, and the gram positive *B. subtilis*, share
 5 more than 50% identity in amino acid sequence within the N-terminal 180 residues
 containing the ATP-binding domain (Fig. 2). Two highly conserved regions (shown
 in bold in Fig. 2) were used to design oligonucleotide primers for application of the
 polymerase chain reaction to *T.th.* genomic DNA. The expected PCR product,
 including the restriction sites (i.e. before cutting) is 345 nucleotides. Use of these
 10 primers with genomic *T.th.* DNA resulted in a product of the expected size. The PCR
 product was then radiolabelled and used to probe genomic DNA in a Southern
 analysis (Fig. 3). Genomic DNA was digested with several different restriction
 endonucleases, electrophoresed in a native agarose gel and then probed with the PCR
 fragment. The Southern analysis showed an *XbaI* fragment of approximately 4 kb,
 15 more than sufficient length to encode the *dnaX* gene. Other restriction nucleases
 produced fragments that were significantly longer, or produced two or more fragments
 indicating presence of a site within the coding sequence of *dnaX*.

To obtain full length *dnaX*, genomic DNA was digested with *XbaI* and ligated into
 20 *XbaI* digested Alter-1 vector. Ligated DNA was transformed into DH5 alpha cells,
 and colonies were screened with the labeled PCR probe. Plasmid DNA was prepared
 from 20 positive colonies and analyzed for the appropriate sized insert using *XbaI*.
 Six of the twenty clones contained the expected 4 kb *XbaI* fragment as an insert, the
 sequence of which is shown in Figs. 4A and 4B.

25 The frameshift site

The *dnaX* gene of *E. coli* produces two proteins, the γ and τ subunits, by a -1
 frameshift (Tsuchihashi and Kornberg, 1990; Flower and McHenry, 1990; Blinkowa
 and Walker, 1990). The full length product yields τ , and the frameshift results in

addition of one amino acid before encountering a stop codon to produce γ . The -1 frameshift site in the *E. coli* *dnaX* gene contains the sequence, A AAA AAG, which follows the X XXY YYZ rule found in retroviral genes (Jacks et. al., 1988). This "slippery sequence" preserves the initial two residues of the tRNAs in the aminoacyl and peptidyl sites both before and after the frameshift. Mutagenesis of the *E. coli* *dnaX* frameshifting site has shown that the first three residues can be nucleotides other than A, but that A's in the second set of three nucleotides is important to frameshifting (Tsuchihashi and Brown, 1992).

Immediately downstream of the stop codon is a potential stem-loop structure which enhances frameshifting, presumably by causing the ribosome to pause. Further, the AAG codon lacks a cognate tRNA in *E. coli* and thus the G residue may facilitate the pause, and has been shown to aid the vigorous frameshifting observed in the *E. coli* *dnaX* gene (Tsuchihashi and Brown, 1992). A fourth component of frameshifting in the *E. coli* *dnaX* gene is presence of an upstream Shine-Dalgarno sequence which is thought to pair with the 16S rRNA to increase the frequency of frameshifting still further (Larsen et. al., 1994).

Examination of the *T.th.* *dnaX* sequence reveals a single site that fulfills the X XXY YYZ rule in which positions 4-7 are A residues. The site is unique from that in *E. coli* as all seven residues are A, and the heptanucleotide sequence is flanked by another A residue on each side (i.e. A₉). Surprisingly, the stop codon immediately downstream of this site is in the -2 frame, although there is a stop codon in the -1 frame 28 nucleotides downstream of the -2 stop codon. Indeed, a -2 frameshift would fulfill the requirement that the first two nucleotides of each codon in the peptidyl and aminoacyl sites be conserved during either a -1 or a -2 frameshift. As with the case of *E. coli* *dnaX*, there are secondary structure step loop structures immediately downstream. Finally, there is a Shine-Dalgarno sequence immediately adjacent to the frameshift site, as well as another Shine-Dalgarno sequence 22 nucleotides upstream of the frameshift site.

Assuming the first stop codon is utilized (i.e. -2 frameshift), the predicted size of the γ subunit in *T.th.* is 454 amino acids for a mass of 49.8 kDa, over 2 kDa larger than the 431 residue γ subunit (47.5 kDa) of *E. coli*. This would result in 2 residues after the -2 frameshift (i.e. after the GluLysLys, the residues LysAla would be added) to be compared to the result of the -1 frameshift in *E. coli* which also results in 2 residues (LysGlu). In the event that a -1 frameshift were utilized in the *T.th. dnaX* gene, then an additional 12 residues would be added following the frameshift for a molecular mass of 50.8 kDa (i.e. after the GluLysLys, the residues LysProAspProLysAlaProProGlyProThrSer would be added). As explained later, this nucleotide sequence was found to promote both -1 and -2 frameshifting in *E. coli* (Fig. 8). But first, we examined *T.th.* cells by Western analysis for the presence of two subunits homologous to *E. coli* γ and τ .

EXAMPLE 2

Frameshifting analysis of the *T.th. dnaX* gene

- 15 Frameshifting was analyzed by inserting the frameshift site into lacZ in the three different reading frames, followed by plating on *X-gal* and scoring for blue or white colony formation (Weiss et. al., 1987). The frameshifting region within *T.th. dnaX* was subcloned into the EcoRI/BamHI sites of pUC19. These sites are within the polylinker inside of the β -galactosidase gene. Three constructs were produced such
- 20 that the insert was either in frame with the downstream coding sequence of β -galactosidase, or were out of frame (either -1 or -2). An additional three constructs were designed by mutating the frameshift sequence and then placing this insert into the three reading frames of the β -galactosidase gene. These six plasmids were constructed as described below.
- 25 The upstream primer for the shifty sequences was 5'-gcg cgg atc cgg agg gag aaa aaa aaa gcc tca gcc ca-3'. The BamHI site for cloning into pUC is underlined. Also, the stop codon, tga, has been mutated to tca (also underlined). The upstream primer for

- the mutant shift sequence was: 5'-gcg cgg atc cgg agg gag aga aga aaa gcc tca gcc ca-3'. The mutant sequence contains two substitutions of a G for an A residue in the polyA stretch (underlined). Three downstream primers were utilized with each upstream primer to create two sets of three inserts in the 0 frame, -1 frame and -2 frame. The sequence of these primers, and the length of insert (after cutting with EcoRI and BanHI and inserting into pUC19) are as follows: 5'-gaa tta aat tcg cgc ttc ggg agg tgg g-3' (0 frameshift, total 58 nucleotide insert); 5'-gcg cga att cgc gct tcg gga ggt ggg-3' (-1 frame, 54mer insert); and 5'-gcg cga att cgg gcg ctt cag gag gtg gg-3' (-2 frame, 56mer insert). The downstream primers have an EcoRI site (underlined); the EcoRI site of the 0 frame insert was blunt ended to produce the greater length insert (converting the EcoRI site to an aattaatt sequence). Also, the tcg sequence, which produces the tga stop codon (underlined) was mutated to tca in the -2 downstream primer so that readthrough would be allowed after the frameshift occurred.
- 15 In summary, a region surrounding the frameshift site and ending at least 5 nucleotides past the -1 frameshift stop codon was inserted into the β galactosidase gene of pUC19 in the three different reading frames (stop codons were mutated to prevent stoppage following a frameshift). These three plasmids were introduced into *E. coli* and plated with *X-gal*. The results, in Fig. 8, show that blue colonies were observed after 24 h
- 20 incubation with all three plasmids and therefore both -1 and -2 frameshifting had occurred.

To further these results, two γ residues were introduced into the polyA tract which should disrupt the ability of this sequence to direct frameshifts. The mutated slippery sequence was inserted into pUC19 followed by transformation into *E. coli* and plating

25 on *X-gal*. The results showed that both -1 and -2 frameshifting was prevented, further supporting the fact that frameshifting requires the polyA tract as expected (Fig. 8).

EXAMPLE 3Expression vector for *T.th.* γ and τ

The *dnaX* gene was cloned into the pET16 expression vector in the steps shown in Fig. 9. First, the bulk of the gene was cloned into pET16 by removing the PmlI/XbaI fragment from pAlter*dnaX*, and placing it into SmaI/XbaI digested Puc19 to yield Puc19*dnaX*Cterm. The N-terminal sequence of the *dnaX* gene was then reconstructed to position an NdeI site at the N-terminus. This was performed by amplifying the 5' region encoding the N-terminal section of γ/τ using an upstream primer containing an NdeI site that hybridizes to the *dnaX* gene at the initiating gtg codon (i.e. to encode Met where the Met is created by the PCR primer, and the Val is the initiating gtg start codon of *dnaX*). The primer sequence for this 5' end was: 5'-gtggtgcatatg gtg agc gcc ctc tac cgc c-3' (where the NdeI site is underlined, and the coding sequence of *dnaX* follows). The downstream primer hybridizes past the PmlI site at nucleotide positions 987 - 1004 downstream of the initiating gtg (primer sequence: 5'-gtggtggtcgac cca gga ggg cca cct cca g-3' where the initial 12 nucleotides contain a SalGI restriction site, followed by the sequence from the region downstream the stop codon). The 1.1 kb nucleotide PCR product was digested with PmlI/NdeI and the PmlI/NdeI fragment was ligated into NdeI/PmlI digested Puc19*dnaX*Cterm to form Puc19*dnaX*. The Puc19*dnaX* plasmid was then digested with NdeI and SalI and the 1.9 kb fragment containing the *dnaX* gene was purified using the Sephaglas BandPrep Kit (Pharmacia-LKB). pET16b was digested with NdeI and XhoI. Then the full length *dnaX* gene was ligated into the digested pET16b to form pET*dnaX*.

EXAMPLE 4 - Expression of *T.th.* γ and τ

As discussed in the previous example, the *dnaX* gene was engineered into the T7 based IPTG inducible pET16 vector such that the initiation codon was placed precisely following the Met residue N-terminal leader sequence (Fig. 9). This should produce a protein containing the entire sequence of γ and τ , along with a 21 residue

leader containing 10 contiguous His residues (tagged- τ = 60.6 kDa; tagged- γ = 52.4 kDa for -2 frameshift). The pETdnaX plasmid was introduced into BL21(DE3)pLysS cells harboring the gene encoding T7 RNA polymerase under control of the lac repressor. Log phase cells were induced with IPTG and analyzed before and after

5 induction in an SDS polyacrylamide gel (Fig. 10, lanes 1 and 2). The result shows that upon induction, two new proteins are expressed with the approximate sizes expected of the *T.th.* γ and τ subunits (larger than *E. coli* γ , and smaller than *E. coli* τ). The two proteins are produced in nearly equal amounts, similar to the case of the *E. coli* γ and τ subunits. Western analysis using antibodies against the *E. coli* γ and τ

10 subunits cross reacted with the induced proteins further supporting their identity as *T.th.* γ and τ (data not shown, but repeated with the pure subunits shown in Fig. 10, lane 6).

EXAMPLE 5

Purification of *T.th.* γ and τ

15 The His-tagged *T.th.* γ and τ proteins were purified from 6 L of induced *E. coli* cells containing the pETdnaX plasmid. Cells were lysed, clarified from cell debris by centrifugation and the supernatant was applied to a HiTrap chelate affinity column. Elution of the chelate affinity column yielded approximately 35 mg of protein in which the two predominant bands migrated in a region consistent with the molecular

20 weight predicted from the *dnaX* gene (Fig. 10, lane 3), and produced a positive signal by Western analysis using polyclonal antibody directed against the *E. coli* γ and τ subunits (lane 4). The γ and τ subunits are present in nearly equal amounts consistent with the nearly equal expression of these proteins in *E. coli* cells harboring the pETdnaX plasmid.

25 The γ and τ subunits were further purified by gel filtration on a Superose 12 column (Fig. 10, lane 4; Fig. 11). Recovery of *T.th.* γ and τ subunits through gel filtration was 81%. The *E. coli* γ and τ subunits, when separated from one another, elute

during gel filtration as tetramers. A mixture of *E. coli* γ/τ results in a mixed tetramer of $\gamma_2\tau_2$ along with τ_4 and γ_4 tetramers (Onrust et. al., 1995). The mixture of *T.th.* γ/τ elutes ahead of the 150 kDa marker, and thus is consistent with the expected mass of a $\gamma_2\tau_2$ tetramer (225 kDa) and τ_4 and γ_4 tetramers.

- 5 As described earlier, the *dnaX* frameshifting sequence could produce either a -1 or -2 framehift to yield a His-tagged γ subunit of mass either 53.3 kDa or 52.4 kDa, respectively. The difference in these two possible products is too close to determine from migration in SDS gels. It also remains possible that two γ products are present and do not resolve under the conditions used. The exact protocol for this purification
- 10 is described below.

- Six liters of BL21(DE3)pLysSpETdnaX cells were grown in LB media containing 50 $\mu\text{g/ml}$ ampicillin and 25 $\mu\text{g/ml}$ chloramphenicol at 37°C to an O.D. of 0.8 and then IPTG was added to a concentration of 2 mM. After a further 2 h at 37°C, cells were harvested by centrifugation and stored at -70°C. The following steps were performed
- 15 at 4°C. Cells (15 g wet weight) were thawed and resuspended in 45 ml 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris HCl (final pH 7.5)) using a dounce homogenizer to complete cell lysis and 450 ml of 5% polyamine P (Sigma) was added. Cell debris was removed by centrifugation at 18,000 rpm for 30 min. in a Sorvall SS24 rotor at 4°C. The supernatant (Fraction I, 40 ml, 376 mg protein) was
- 20 applied to a 5 ml HiTrap Chelating Separose column (Pharmacia-LKB). The column was washed with 25 ml of binding buffer, then with 30 ml of binding buffer containing 60 mM imidazole, and then eluted with 30 ml of 0.5 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5). Fractions of 1 ml were collected and analyzed on an
- 25 8% Coomassie Blue stained SDS polyacrylamide gel. Fractions containing subunits migrating at the *T.th.* γ and τ positions, and exhibiting cross reactivity with antibody to *E. coli* γ and τ in a Western analysis, were pooled and dialyzed against buffer A (20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT and 10% glycerol) containing 0.5 M NaCl (Fraction II, 36 mg in 7 ml). Fraction II was diluted 2-fold with buffer A

and passed through a 2 ml ATP agarose column equilibrated in buffer A containing 0.2 M NaCl to remove any *E. coli* γ complex contaminant. Then 0.18 mg (300 ml) Fraction II was gel filtered on a 24 ml Superose 12 column (Pharmacia-LKB) in buffer A containing 0.5 M NaCl. After the first 216 drops, fractions of 200 μ l were collected (Fraction III) and analyzed by Western analysis (by procedures similar to those described in Example 6), by ATPase assays and by Coomassie Blue staining of an 8% Coomassie Blue stained SDS polyacrylamide gel. The Coomassie stained gels and Western analysis of recombinant *T.th. gamma and tau* for these purification steps are summarized in Fig. 10.

EXAMPLE 6

Western Analysis of *T.th.* cells for presence of γ and τ subunits

Polyclonal antibody to *E. coli* γ/τ - *E. coli* γ subunit was prepared as described (Studwell-Vaughan and O'Donnell, 1991). Pure γ subunit (100 μ g) was brought up in Freund's adjuvant and injected subcutaneously into a New Zealand Rabbit (Poccono Rabbit Farms). After two weeks, a booster consisting of 50 μ g γ in Freund's adjuvant was administered, followed after two weeks by a third injection (50 μ g).

The homology between the amino terminal regions of *T.th.* and *E. coli* γ/τ subunits suggested that there may be some epitopes in common between them. Hence, polyclonal antibody directed against the *E. coli* γ/τ subunits was raised in rabbits for use in probing *T.th.* cells by Western analysis. Fig. 7 shows the results of a Western analysis of whole *T.th.* cells lysed in SDS. The results show that in *T.th.* cells, the antibody is rather specific for two high molecular proteins which migrate in the vicinity of the molecular masses of *E. coli* γ and τ subunits.

Procedure for Western Analysis

Samples were analyzed in duplicate 10 % SDS polyacrylamide gels by the Western method (Towbin et. al. 1979). One gel was Coomassie stained to evaluate the pattern of proteins present, and the other gel was then electroblotted onto a nitrocellulose membrane (Schleicher and Schuell). For molecular size markers, the kaliedoscope molecular weight markers (Bio-Rad) were used to verify by visualization that transfer of proteins onto the blotted membrane had occurred. The gel used in electroblotting was also stained after electroblotting to confirm that efficient transfer of protein had occurred. Membranes were blocked using 5% non-fat milk, washed with 0.05% Tween in TBS (TBS-T) and then incubated for over 1 h with a 1/5000 dilution of rabbit polyclonal antibody directed against *E. coli* γ and τ in 1 % gelatin in TBS-T at room temperature. Membranes were washed using TBS-T buffer and then antibody was detected on X-ray film (Kodak) by using the ECL kit from (Amersham) and the manufactures recommended procedures.

Samples included: 1) a mixture of *E. coli* γ (15 ng) and τ (15 ng) subunits; 2) *T.th.* whole cells (100 μ l) suspended in cracking buffer; and 3) purified *T.th.* γ and τ fraction II (0.6 μ g as a mixture).

EXAMPLE 7

Characterization of the ATPase Activity of γ/τ -

The *E. coli* τ subunit is a DNA dependent ATPase (Lee and Walker, 1987; Tsuchihashi and Kornberg, 1989). The γ subunit binds ATP but does not hydrolyze it even in the presence of DNA unless other subunits of the DNA polymerase III holoenzyme are also present (Onrust et. al., 1991). Next we examined the *T.th.* γ/τ subunits for DNA dependent ATPase activity. The γ/τ preparation was, in fact, a DNA stimulated ATPase (Fig. 11, top panel). The specific activity of the *T.th.* γ/τ was 11.5 mol ATP hydrolyzed/mol γ/τ (as monomer and assuming an equal mixture of the two). Furthermore, analysis of the gel filtration column fractions shows that the ATPase activity coelutes with the *T.th.* γ/τ subunits, supporting evidence that the weak ATPase activity is intrinsic to the γ/τ subunits (Fig. 11). The specific activity of the

γ/τ preparation before gel filtration was the same as after gel filtration (within 10%), further indicating that the DNA stimulated ATPase is an inherent activity of the γ/τ subunits. Presumably, only the τ subunit contains ATPase activity, as in the case of *E. coli*. Assuming only *T.th.* τ contains ATPase activity, its specific activity is twice the observed rate (after factoring out the weight of γ). This rate is still only one-fifth that of *E. coli* τ .

The *T.th.* γ/τ ATPase activity is lower at 37°C than at 65°C (middle panel), consistent with the expected behavior of protein activity from a thermophilic source. However, there is no apparent increase in activity in proceeding from 50°C to 65°C (the rapid breakdown of ATP above 65°C precluded measurement of ATPase activity at temperatures above 65°C). In contrast, the *E. coli* τ subunit lost most of its ATPase activity upon elevating the temperature to 50°C (middle panel). These reactions contain no stabilizers such as a nonionic detergent or gelatin, nor did they include substrates such as ATP, DNA or magnesium.

Last, the relative stability of *T.th.* γ/τ and *E. coli* γ/τ to addition of NaCl (Fig. 12, bottom panel) was examined. Whereas the *E. coli* τ subunit rapidly lost activity at even 0.2 M NaCl, the *T.th.* γ/τ retained full activity in 1.0 M NaCl and was still 80 % active in 1.5 M NaCl. The detailed procedure for the ATPase activity assay is described below.

ATPase assays: ATPase assays were performed in 20 μ l of 20 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$ containing 0.72 μ g of M13mp18 ssDNA (where indicated), 100 mM [γ - ^{32}P]-ATP (specific activity of 2000-4000 cpm/pmol), and the indicated protein. Some reactions contained additional NaCl where indicated. Reactions were incubated at the temperatures indicated in the figure legends for 30 min. and then were quenched with an equal volume of 25 mM EDTA (final). The aliquots were analyzed by spotting them (1 μ l each) onto thin layer chromatography (TLC) sheets coated with Cel-300 polyethyleneimine (Brinkmann Instruments Co.). TLC sheets were

developed in 0.5 M lithium chloride, 1 M formic acid. An autoradiogram of the TLC chromatogram was used to visualize Pi at the solvent front and ATP near the origin which were then cut from the TLC sheet and quantitated by liquid scintillation. The extent of ATP hydrolyzed was used to calculate the mol of Pi released per mol of protein per min. One mol of *E. coli* τ was calculated assuming a mass of 71 kDa per monomer. The *T.th.* γ and τ preparation was treated as an equal mixture and thus one mole of protein as monomer was the average of the predicted masses of the γ and τ subunits (54 kDa).

EXAMPLE 7

- 10 Western analysis of *T.th.* cells for presence of γ and τ subunits - The homology between the amino terminal regions of *T.th.* and *E. coli* γ/τ subunits suggested that there may be some epitopes in common between them. Hence, polyclonal antibody directed against the *E. coli* γ/τ subunits was raised in rabbits for use in probing *T.th.* cells by Western analysis. Fig. 7 shows the results of a Western analysis of whole
- 15 *T.th.* cells lysed in SDS. The results show that in *T.th.* cells, the antibody is rather specific for two high molecular proteins which migrate in the vicinity of the molecular masses of *E. coli* γ and τ subunits.

EXAMPLE 8

Homolog of *T.th.* γ/τ to *dnaX* gene products of other organism

- 20 The XbaI insert encoded an open reading frame, starting with a GTG codon, of 529 amino acids in length (58.0 kDa), closer to the predicted length of the *B. subtilis* τ subunit (563 amino acids, 62.7 kDa mass)(Alonso et. al., 1986) than the *E. coli* τ subunit (71.1 kDa)(Yin et. al., 1986). *dnaX* encoding the γ/τ subunits of *E. coli* DNA polymerase III holoenzyme is homologous to the *holB* gene encoding the δ' subunit of
- 25 the γ complex clamp loader, and this homology extends to all 5 subunits of the eukaryotic RFC clamp loader as well as the bacteriophage gene protein 44 of the

gp44/62 clamp loading complex (O'Donnell et. al., 1993). These gene products show greatest homology over the N-terminal 166 amino acid residues (of *E. coli* dnaX); the C-terminal regions are more divergent. Fig. 4 shows an alignment of the amino acid sequence of the N-terminal regions of the *Tth* dnaX gene product to those of several other bacteria. The consensus GXXGXGKT motif for nucleotide binding, is conserved in all these protein products. Further, the *E. coli* δ' crystal structure reveals one atom of zinc coordinated to four Cys residues (Guenther, 1996). These four Cys residues are conserved in the *E. coli* dnaX gene, and the γ and τ subunits encoded by *E. coli* dnaX bind one atom of zinc (J. Turner and M. O'Donnell, unpublished). These Cys residues are also conserved in *Tth* dnaX (shown in Fig. 4). Overall, the level of amino acid identity relative to *E. coli* dnaX in the N-terminal 165 residues of *Tth* dnaX is 53 %. The *Tth* dnaX gene is just as homologous to the *B. subtilis* dnaX (53 % identity) gene relative to *E. coli* dnaX. After this region of homology, the C-terminal region of *Tth* dnaX shares 26% and 20% identity to *E. coli* and *B. subtilis* dnaX, respectively. A proline rich region, downstream of the conserved region, is also present in *Tth* dnaX (residues 346-375), but not in the *B. subtilis* dnaX (see Figs. 3A and 3B). The overall identity between *E. coli* dnaX and *Tth* dnaX over the entire gene is 34%. Identity of *Tth*. dnaX to *B. subtilis* dnaX over the entire gene is 28%.

Comparison of dnaX genes from *T.th.* and *E. coli*

The above identifies a homologue of the *dnaX* gene of *E. coli* in *Thermus thermophilus*. Like the *E. coli* gene, *T.th. dnaX* encodes two related proteins through use of a highly efficient translational frameshift. The *T.th.* γ/τ subunits are tetramers, or mixed tetramers, similar to the γ and τ subunits of *E. coli*. Further, the γ/τ subunit is a DNA stimulated ATPase like its *E. coli* counterpart. As expected for proteins from a thermophile, the *T.th.* γ/τ ATPase activity is thermostabile and resistant to added salt.

In *E. coli*, γ is a component of the clamp loader, and the τ subunit serves the function of holding the clamp loading apparatus together with two DNA polymerases for coordinated replication of duplex DNA. The presence of γ in *T.th.* suggests it has a clamp loading apparatus and thus a clamp as well. The presence of the τ subunit *T.th.* implies that *T.th.* contains a replicative polymerase with a structure similar to that of *E. coli* DNA polymerase III holoenzyme.

A significant difference between *E. coli* and *T.th.* *dnaX* genes is in the translational frameshift sequence. In *E. coli*, the heptamer frameshift site contains six A residues followed by a G residue in the context A AAA AAG. This sequence satisfies the X XXY YYZ rule for -1 frameshifting. The frameshift is made more efficient by the absence of the AAG tRNA for Lys which presumably leads to stalling of the ribosome at the frameshift site and increases the efficiency of frameshifting (Tsuchihashi and Brown, 1992). Two additional aids to frameshifting include a downstream hairpin, and an upstream Shine-Dalgarno sequence (Tsuchihashi and Kornberg, 1990; Larsen et. al., 1994). The -1 frameshift leads to incorporation of one unique residue at the C-terminus of *E. coli* γ before encounter with a stop codon.

In *T.th.*, the *dnaX* frameshifting heptamer is A AAA AAA, and it is flanked by two other A residues, one on each side. There is also a downstream region of secondary structure. The nearest downstream stop codon is positioned such that gamma would contain only one unique amino acid, as in *E. coli*. However, the *T.th.* stop codon is in the -2 reading frame thus requires a -2 frameshift. No precedent exists in nature for -2 frameshifting, although -2 frameshifting has been shown to occur in test cases (Weiss et. al., 1987). *In vivo* analysis of the *T.th.* frameshift sequence shows that this natural sequence promotes both -1 and -2 frameshifting in *E. coli*. Whereas the -2 frameshift results in only one unique C-terminal residue, a -1 frameshift would result in an extension of 12 C-terminal residues. At present, the results do not discriminate which path occurs in *T.th.*, a -1 or -2 frameshift, or a combination of the two.

There are two Shine-Dalgarno sequences just upstream of the frameshift site in *T.th. dnaX*. In two cases of frameshifting in *E. coli*, an upstream Shine-Dalgarno sequence has been shown to stimulate frameshifting (reviewed in Weiss et. al., 1997). In release factor 2 (RF2), the Shine-Dalgarno is 3 nucleotides upstream of the shift site, and it stimulates a +1 frameshift event. In the case of *E. coli dnaX*, a Shine-Dalgarno sequence 10 nucleotides upstream of the shift sequence stimulates the -1 frameshift. One of the *T.th. dnaX* Shine-Dalgarno sequences is immediately adjacent to the frameshift sequence with no extra space, the other is 22 residues upstream of the frameshift site. Which of these Shine-Dalgarno sequences plays a role in *T.th. dnaX* frameshifting, if any, will require future study.

In *E. coli*, efficient separation of the two polypeptides, γ and τ , is achieved by mutation of the frameshift site such that only one polypeptide is produced from the gene (Tsuchihashi and Kornberg, 1990). Substitution of G-to-A in two positions of the heptamer of *T.th. dnaX* eliminates frameshifting and thus should be a source to obtain τ subunit free of γ . To produce pure γ subunit free of τ , the frameshifting site and sequence immediately downstream of it can be substituted for an in frame sequence with a stop codon.

Examination of the *B. subtilis dnaX* gene shows no frameshift sequence that satisfies the X XXY YYZ rule. Hence, it would appear that *dnaX* does not make two proteins in this gram positive organism.

Rapid thermal motions associated with high temperature may make coordination of complicated processes more difficult. It seems possible that organizing the components of the replication apparatus may become yet more important at higher temperature. Hence, production of a τ subunit that could be used to crosslink two polymerases and a clamp loader into one organized particle may be most useful at elevated temperature.

- As stated above, the following examples describe the continued isolation and purification of the substantial entirety of the Polymerase III from the extreme thermophile *Thermus thermophilus*. It is to be understood that the following exposition is reflective of the protocol and characteristics, both morphological and functional, of the Polymerase III-type enzymes that are the focus of the present invention, and that the invention is hereby illustrated and comprehends the entire class of enzymes of thermophilic origin.

EXAMPLE 9

Purification of the *Thermus thermophilus* DNA polymerase III

- 10 All steps in the purification assay were performed at 4°C. The following assay was used in the purification of DNA polymerase from *T.th.* cell extracts. Assays contained 2.5 mg activated calf thymus DNA (Sigma Chemical Company) in a final volume of 25 ml of 20 mM Tris-Cl (pH 7.5), 8 mM MgCl₂, 5 mM DTT, 0.5 mM EDTA, 40 mg/ml BSA, 4% glycerol, 0.5 mM ATP, 3 mM each dCTP, dGTP, dATP, and 20 mM
- 15 [α -³²P]dTTP. An aliquot of the fraction to be assayed was added to the assay mixture on ice followed by incubation at 60°C for 5 min. DNA synthesis was quantitated using DE81 paper followed by washing off unincorporated nucleotide. Incorporated nucleotide was determined by scintillation counting of the filters.

- Thermus thermophilus cell extracts were prepared by suspending 35 grams of cell
- 20 paste in 200 ml of 50 mM TRIS-HCl, pH=7.5, 30 mM spermidine, 100 mM NaCl, 0.5 mM EDTA, 5 mM DTT, 5% glycerol, followed by disruption by passage through a French pressure cell (15,000 PSI). Cell debris was removed by centrifugation (12,000 RPM, 60 min). DNA polymerase III in the clarified supernatant was precipitated by treatment with ammonium sulphate (0.226 gm/liter) and recovered by centrifugation.
- 25 This fraction was then backwashed with the same buffer (but lacking spermidine) containing 0.20 gm/l ammonium sulfate. The pellet was then resuspended in buffer A

and dialyzed overnight against 2 liters of buffer A; a precipitate which formed during dialysis was removed by centrifugation (17,000 RPM, 20 min).

The clarified dialysis supernatant, containing approximately 336 mg of protein, was applied onto a 60 ml heparin agarose column equilibrated in buffer A which was washed with the same buffer until A280 reached baseline. The column was developed with a 500 ml linear gradient of buffer A from 0 to 500 mM NaCl. More tightly adhered proteins were washed off the column by treatment with buffer A (20 mM Tris Hcl, pH = 7.5, 0.1 mM EDTA, 5mM DTT, and 10% glycerol) and 1M NaCl. Some DNA polymerase activity flowed through the column. Two peaks (HEP.P1 and HEP.P2) of DNA polymerase activity eluted from the heparin agarose column containing 20 mg and 2 mg of total protein respectively (Fig. 13A). These were kept separate throughout the remainder of the purification protocol.

The Pol III resided in HEP.P1 as indicated by the following criteria: 1) Western analysis using antibody directed against the α subunit of *E. coli* Pol III indicated presence of Pol III in HEP.P1, 2) Only the HEP.P1 fraction was capable of extending a single primer around an M13mp18 7.2 kb ssDNA circle (explained later in Example 14). This type of long primer extension is a characteristic of Pol III type enzymes. 3) Only the HEP.P1 provided DNA polymerase activity that was retained on an ATP-agarose affinity column. This is indicative of a Pol III-type DNA polymerase since the γ and τ subunits are ATP interactive proteins.

The first peak of the heparin agarose column (HEP.P1: 20 mg in 127.5 ml) was dialyzed against buffer A and applied onto a 2ml N6-linkage ATP agarose column pre-equilibrated in the same buffer. Bound protein was eluted by a slow (0.05 ml/min) wash with buffer A + 2M NaCl and collected into 200 μ l fractions. Chromatography of peak HEP.P1 yielded a flow-through (HEP.P1-ATP-FT) and a bound fraction (HEP.P1-ATP-Bound) (Fig. 13B). Binding of peak HEP.P2 to the

ATP column could not be detected, though DNA polymerase activity was recovered in the flow-through.

- The HEP.P1-ATP-Bound fractions from the ATP agarose chromatographic step were further purified by anion exchange over monoQ. The HEP.P1-ATP-Bound fractions were diluted with buffer A to approximately the conductivity of buffer A plus 25 mM NaCl and applied to a 1ml monoQ column equilibrated in Buffer A. DNA polymerase activity eluted in the flow-through and in two resolved chromatographic peaks (MONOQ peak1 and peak2) (Fig. 13C). Peak 2 was by far the major source of DNA polymerase activity. Western analysis using rabbit antibody directed against the *E. coli* α subunit confirmed presence of the α subunit in the second peak (see the Western analysis in Fig. 14B). Antibody against the *E. coli* γ subunit also confirmed the presence of the γ subunit in the second peak (not shown). Some reaction against α and γ was also present in the minor peak (first peak). The Coomassie Blue SDS polyacrylamide gel of the MonoQ fractions (Fig. 14A) showed a band that co-migrated with *E. coli* α and was in the same position as the antibody reactive material (antibody against *E. coli* α). Also present are bands corresponding to τ , δ and δ' . These subunits, along with β , are all that is necessary for rapid and processive synthesis and primer extension over a long (> 7 kb) stretch of ssDNA in the case of *E. coli* DNA Polymerase III holoenzyme.
- The Pol III-type enzyme purified from *T.th.* may be a Pol III*-like enzyme that contains the DNA polymerase and clamp loader subunits (*i.e.* like the Pol III* of *E. coli*). The evidence for this is: 1) the presence of *dnaX* and *dnaE* gene products in the same column fractions as indicated by Western analysis (see above); 2) the ability of this enzyme to extend a primer around a 7.2 kb circular ssDNA upon adding only β (see Example 14); 3) stimulation of Pol III by adding β on linear DNA, indicating β subunit is not present in saturating amounts (see Example 13); and 4) the presence of τ in *T.th.* which may glue the polymerase and clamp loader into a Pol III* as in *E.*

coli; and 5) the comigration of α with subunits γ , δ and δ' of the clamp loader in the column fractions of the last chromatographic step (MonoQ, Fig. 14A)..

Micro-sequencing of *T. th* DNA Polymerase III α subunit

The α subunit from the purified *T. th* DNA polymerase III

- 5 (HEP.P1.ATP-Bound.MONOQ peak2) was blotted onto PVDF membrane and was cut out of the SDS-PAGE gel and submitted to the Protein-Nucleic Acid Facility at Rockefeller University for N-terminal sequencing and proteolytic digestion, purification and microsequencing of the resultant peptides. Analysis of the a candidate band ($M_w \approx 130\text{kD}$) yielded four peptides, two of which (TTH1, TTH2)
- 10 showed sequence similarity to α subunits from various bacterial sources (see Fig. 15).

EXAMPLE 10

Identification of the *Thermus thermophilus* *dnaE* gene encoding the α subunit of DNA polymerase III holoenzyme

- Cloning of the *dnaE* gene was started with the sequence of the TTH1 peptide from the
- 15 purified α subunit (FFIEIQNHGLSEQK). The fragment was aligned to a region at approximately 180 amino acids downstream of the N-termini of several other known α subunits as shown in Fig. 15. The upstream 33mer (5'-GTGGGATCCGTGGTTCTGGATCTCGATGAAGAA-3') consists of a BamHI site within the first 9 nucleotides (underlined) and the sequence coding for the
- 20 following peptide HGLSEQK on the complementary strand. The downstream 29mer (5'-GTGGGATCCACGGSTSTCSGAGCAGAAG-3') consists of a BamHI site within the first 9 nucleotides (underlined) and the following sequence coding for the peptide FFIEIQNH.

- These two primers were directed away from each other for the purpose of performing
- 25 inverse PCR (also called circular PCR). The amplification reactions contained 10ng *T. th.* genomic DNA (that had been cut and religated with XmaI), 0.5 mM of each primer, in a volume of 100 μl of Vent polymerase reaction mixture containing 10 μl

ThermoPol Buffer, 0.5 mM of each dNTP and 0.25 mM MgSO₄. Amplification was performed using the following cycling scheme:

1. 4 cycles of: 95.5°C - 30", 45°C - 30", 75°C - 8'
2. 6 cycles of: 95.5°C - 30", 50°C - 30", 75°C - 6'
- 5 3. 30 cycles of: 95.5°C - 30", 52.5°C - 30", 75°C - 5'

A 1.4kb fragment was obtained and cloned into pBS-SK:BamHI (i.e. pBS-SK (Stratagene) was cut with BamHI). This sequence was bracketted by the 29mer primer on both sides and contained the sequence coding for the N-terminal part of the α subunit up to the peptide used for primer design.

- 10 To obtain further *dnaE* gene sequence, the *TTH2* peptide was used. It was aligned to a region about 600 amino acids from the N-termini of the other known α subunits (Fig. 15B).

- The upstream 34mer (5'-GCGGGATCCTCAACGAGGAGCTCTCCATCTTCAA-3') consists of a BamHI site within the first 9 nucleotides (underlined) and the sequence
- 15 from the end of the fragment previously obtained. The downstream 33mer (5'-GCGGGATCCTTGTCTCGTCSAGSGTSAGSGCGTCGTA-3') consists of a BamHI site within the first 9 nucleotides (underlined) and the following sequence coding for the peptide YDALTLDD on the complementary strand. The amplification reactions contained 10 ng *T.th.* genomic DNA, 0.5 mM of each primer, in a volume of 100 μ l of
 - 20 Vent polymerase reaction mixture containing 10 μ l ThermoPol Buffer, 0.5 mM of each dNTP and 0.25 mM Mg SO₄. Amplification was performed using the following cycling scheme:

1. 4 cycles of: 95.5°C - 30", 45°C - 30", 75°C - 8'
2. 6 cycles of: 95.5°C - 30", 50°C - 30", 75°C - 6'
- 25 3. 30 cycles of: 95.5°C - 30", 55°C - 30", 75°C - 5'

A 1.2kb PCR fragment was obtained and cloned into pUC19:BamHI. The fragment was bracketted by the downstream primer on both sides and contained the region overlapping in 56 bp with the fragment previously cloned.

To obtain yet more *dnaE* sequence, the following primers were used. The upstream 39mer (3'-GTGTGGATCCTCGTCCCCCTCATGCGCGACCAGGAAGGG-5') consists of a BamHI site within the first 10 nucleotides (underlined) and the sequence from the end of the fragment previously obtained. The downstream 27mer (5'-GTGTGGATCCTTCTTCTTSCCATSGC-3') consists of a BamHI site within the first 10 nucleotides (underlined), and the sequence coding for the peptide AMGKKK (at position approximately 800 residues from the N terminus) on the complementary strand. The AMGKKK sequence was chosen for primer design as it is highly conserved among the known gram-negative α subunits. The amplification reactions contained 10 ng *T.th.* genomic DNA, 0.5 mM of each primer, in a volume of 100 μ l of Taq polymerase reaction mixture containing 10 μ l PCR Buffer, 0.5 mM of each dNTP and 2.5 mM MgCl₂. Amplification was performed using the following cycling scheme:

1. 3 cycles of: 95.5°C - 30", 45°C - 30", 72°C - 8'
2. 6 cycles of: 94.5°C - 30", 55°C - 30", 72°C - 6'
3. 32 cycles of: 94.5°C - 30", 50°C - 30", 72°C - 5'

A 2.3kb PCR fragment was obtained instead of the expected 0.6 kb fragment. BamHI digestion of the PCR product resulted in three fragments of 1.1 kb, 0.7kb and 0.5kb. The 1.1 kb fragment was cloned into pUC19:BamHI. It turned out to be the one adjacent to the fragment previously obtained and contained the *dnaE* sequence right up to the region coding for the AMGKKK peptide, but was disrupted by an intein just upstream of this region.

The sequence that follows this was amplified from the 2.3kb original PCR product using the same conditions and cycling scheme as for the 2.3kb fragment. The

downstream primer was the same as in the previous step. The upstream 27mer (3'-GTGTGGATCCGTGGTGACCTTAGCCAC-5') consisted of a BamHI site within the first 9 nucleotides (underlined) and the sequence from the end of the 1.1kb fragment previously described.

- 5 The expected 1.2kb PCR fragment was obtained and cloned into pUC19:SmaI. This fragment coded for the rest of the intein and the end of it was used to obtain the next sequence of *dnaE* downstream of this region. The upstream 30mer (3'-TTCGTGTCCGAGGACCTTGTGGTCCACAAC-5') was a sequence from the end of the intein. The downstream 23mer
- 10 (5'-CCAGAATCGTCTGCTGGTCGTAG-3') was the sequence from the end of the *dnaE* gene of *D.rad.* (coding on the complementary strand for the region slightly homologous in the distantly related α subunits and possibly highly homologous between *T.th.* and *D.rad.* α subunits). The amplification reactions contained 10 ng *T.th.* genomic DNA, 0.5 mM of each primer, in a volume of 100 μ l of Vent
- 15 polymerase reaction mixture containing 10 μ l ThermoPol Buffer, 0.5 mM of each dNTP and 0.1 mM Mg SO₄. Amplification was performed using the following cycling scheme:

1. 3 cycles of: 95.5°C - 30", 55°C - 30", 75°C - 8'
2. 32 cycles of: 94.5°C - 30", 50°C - 30", 75°C - 5'

- 20 A 2.5kb PCR fragment was obtained and cloned into pUC19:SmaI. This fragment contained the *dnaE* sequence coding for the 300 amino acids next to the AMGKKK region disrupted by yet a second intein inside another sequence that is conserved among the known α subunits (FNKSHSAAY).

To obtain the rest of the *dnaE* gene the upstream 19mer

- 25 (5'-AGCACCTGGAGGAGCTTC-3') from the end of the known *dnaE* sequence was used. The downstream primer was: 5'-CATGTCGTACTGGGTGTAC-3'. The amplification reactions contained 10 ng *T.th.* genomic DNA, 0.5 mM of each primer,

in a volume of 100 µl of Vent polymerase reaction mixture containing 10 µl ThermoPol Buffer, 0.5 mM of each dNTP and 0.1 mM Mg SO₄. Amplification was performed using the following cycling scheme:

1. 3 cycles of: 95.5°C - 30", 55°C - 30", 75°C - 8'
- 5 2. 32 cycles of: 94.5°C - 30", 50°C - 30", 75°C - 5'

A 1.0kb fragment bracketed by this upstream primer was obtained. It contained the 3' end of the *dnaE* gene.

EXAMPLE 11

10 Cloning and Expression of the *Thermus thermophilus dnaQ* gene encoding the ε subunit of DNA polymerase III holoenzyme

Cloning of *dnaQ* - The DnaQ gene of *E. coli* and the corresponding region of PolC of *B. subtilis*, evolutionary divergent organisms, share approximately 30% identity. Comparison of the predicted amino acid sequences encoded by DnaQ of *E. coli* and PolC of *B. subtilis* revealed two highly conserved regions positions (Fig. 17). Within
15 each of these regions, a nine amino acid sequence was used to design two oligonucleotide primers for use in the polymerase chain reaction.

The regions highly conservative among Pol III exonucleases were chosen to design the degenerate primers for the amplification of a *T.th. dnaQ* internal fragment (see Fig. 17). DNA oligonucleotides for amplification of *T.th.* genomic DNA were as
20 follows. The upstream 27mer (5'-GTSGTSNNSGACNNSGAGACSGGG-3') encodes the following sequence (VVXDXETTG). The downstream 27mer (5'-GAASCCSNNGTCGAASNNGGCGTTGTG-3') encodes the sequence HNAXFDXGF on the complementary strand. The amplification reactions contained
25 10 ng *T.th.* genomic DNA, 0.5 mM of each primer, in a volume of 100 µl of Vent polymerase reaction mixture containing 10 µl ThermoPol Buffer, 0.5 mM of each

in a volume of 100 µl of Vent polymerase reaction mixture containing 10 µl ThermoPol Buffer, 0.5 mM of each dNTP and 0.1 mM Mg SO₄. Amplification was performed using the following cycling scheme:

1. 3 cycles of: 95.5°C - 30", 55°C - 30", 75°C - 8'
- 5 2. 32 cycles of: 94.5°C - 30", 50°C - 30", 75°C - 5'

A 1.0kb fragment bracketed by this upstream primer was obtained. It contained the 3' end of the *dnaE* gene.

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25 10 ng *T.th.* genomic DNA, 0.5 mM of each primer, in a volume of 100 µl of Vent polymerase reaction mixture containing 10 µl ThermoPol Buffer, 0.5 mM of each

dNTP and 0.5 mM MgSO₄. Amplification was performed using the following cycling scheme:

1. 5 cycles of: 95.5°C - 30", 40°C - 30", 72°C - 2'
2. 5 cycles of: 95.5°C - 30", 45°C - 30", 72°C - 2'
- 5 3. 30 cycles of: 95.5°C - 30", 50°C - 30", 72°C - 30"

Products were visualized in a 1.5 % native agarose gel. A fragment of the expected size of 270 bp was cloned into the SmaI site of pUC19 and sequenced with the CircumVent Thermal Cycle DNA sequencing kit according to the manufacturer's instructions (New England Biolabs).

- 10 To obtain further sequence of the *dnaQ* gene, genomic DNA was digested with either mhoI, BamHI, KpnI or NcoI. These restriction enzymes were chosen because the cut *T.th.* genomic DNA frequently. 0.1 µg of DNA for each digest was ligated by T4 DNA ligase in 50 µl of ligation buffer (50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 mg/ml bovine serum albumin) overnight at 20°C.
- 15 The ligation mixtures were used for circular PCR.

DNA oligonucleotides for amplification of *T.th.* genomic DNA were the following. The upstream 27mer (5'-CGGGGATCCACCTCAATCACCTCGTGG-3') consists of a BamHI site within the first 9 nucleotides (underlined) and the sequence complementary to 42-61bp region of the previously cloned *dnaQ* fragment. The downstream 30mer (5'-CGGGGATCCGCCACCTTGCGGCTCCGGGTG-3') consists of a BamHI site within the first 9 nucleotides (underlined) and the sequence corresponding to 240-261 bp region of the *dnaQ* fragment (see Fig. 17).

- 20 The amplification reactions contained 1 ng *T.th.* genomic DNA (that had been cut with NcoI and religated into circular DNA for circular PCR), 0.4 mM of each primer,
- 25 in a volume of 100 µl of Vent polymerase reaction mixture containing 10 µl

ThermoPol Buffer, 0.5 mM of each dNTP, 0.5 mM MgSO₄, and 10% DMSO.

Circular amplification was performed using the following cycling scheme:

1. 5 cycles of: 95.5°C - 30", 50°C - 30", 72°C - 8'
2. 35 cycles of: 95.5°C - 30", 55°C - 30", 72°C - 6'
3. 72°C - 10'

A 1.5 kb fragment was obtained and cloned into the BamHI site of the pUC19 vector. Partial sequencing of the fragment revealed that it contained the *dnaQ* regions adjacent to sequences corresponding to the PCR primers and hence contained the sequences both upstream and downstream of the previously cloned *dnaQ* fragment. One of NcoI sites turned out to be approximately 300 bp downstream of the end of the first cloned *dnaQ* sequence and hence did not include the 3' end of *dnaQ*. To obtain the 3' end, another inverse PCR reaction was performed. Since an ApaI restriction site was recognized within this newly sequenced *dnaQ* fragment, the circular PCR procedure was performed using as template an ApaI digest of *T.th.* genomic DNA that was ligated (circularized) under the same conditions as described above.

DNA oligonucleotides for amplification of the ApaI/religated *T.th.* genomic DNA were as follows. The upstream 31mer (5'-GCGCTCTAGACGAGTTCCCAAAGCGTGCGGT-3') consists of a *mbaI* site within the first 10 nucleotides (underlined) and the sequence complementary to the region downstream of the ApaI restriction site in the newly sequenced *dnaQ* fragment. The downstream 31mer (5'-CGCGTCTAGATCACCTGTATCCAGA-3') consists of a *XbaI* site within the first 10 nucleotides (underlined) and the sequence corresponding to another region downstream of the ApaI restriction site in the newly sequenced *dnaQ* fragment. The 1.7 kb PCR fragment was cloned into the *XbaI* site of the pUC19 vector and partially sequenced. The sequence of *dnaQ*, and the protein sequence of the ϵ subunit encoded by it, is shown in Fig. 18.

The *dnaQ* gene is encoded by an open reading frame of 209 (or 190 depending on which Val is used as the initiating residue) amino acids in length (23598.5 kDa - or 21383.8 kDa for shorter version), similar to the length of the *E.coli* ϵ subunit (243 amino acids, 27099.1 kDa mass) (see Fig. 17).

- 5 The entire amino acid sequence of the ϵ subunit predicted from the *T.th.* *dnaQ* gene aligns with the predicted amino acid sequence of the *dnaQ* genes of other organisms with only a few gaps and insertions (the first two amino acids, and four positions downstream) (Fig. 17). The consensus motifs (VVXD $\overline{\text{X}}$ ETT $\overline{\text{G}}$, HNAXFDX $\overline{\text{G}}$ F, and HRALYD), characteristic for exonucleases, are conserved. Overall, the level of
- 10 amino acid identity relative to most of the known ϵ subunits, or corresponding proofreading exonuclease domains of gram positive PolC genes is approximately 30%. Upstream of start 1 (Fig. 17) there were stop codons in all three reading frames.

- Expression of *DnaQ* - The *DnaQ* gene was cloned gene into the pET24-a expression vector in two steps. First, the PCR fragment encoding the N-terminal part of the gene
- 15 was cloned into the pUC19 plasmid, containing the *Apal* inverse PCR fragment into *NdeI*/*Apal* sites. DNA oligonucleotides for amplification of *T.th.* genomic DNA were as follows. The upstream 33mer (5'-GCGGCGCCATATGGTGGTGGTCCTGGACCTGGAG-3') consists of an *NdeI* site within the first 12 nucleotides (underlined) and the beginning of the *dnaQ* gene.
 - 20 The downstream 31mer (5'-CGCGTCTAGATCACCTGTATCCAGA-3'), already used for *Apal* circular PCR, consists of an *XbaI* site within the first 10 nucleotides (underlined) and the sequence corresponding to the region downstream of the *Apal* restriction site. The 2.2 kb *NdeI*/*Sall* fragment was then cloned into the *NdeI*/*XhoI* sites of the pET16 vector to produce pET24-a:*dnaQ*. The ϵ subunit was expressed in
 - 25 the BL21/LysS strain transformed by the pET24-a:*dnaQ* plasmid.

EXAMPLE 12

The *Thermus thermophilus* dnaN gene encoding the β subunit of DNA polymerase III holoenzyme

Strategy of cloning *DnaN* by use of *DnaA* - *DnaN* proteins are highly divergent in bacteria making it difficult to clone them by homology. The level of identity between

5 *DnaN* representatives from *E. coli* and *B. subtilis* is as low as 18%. These 18% of identical amino acid residues are dispersed through the proteins rather than clustering together in conservative regions, further complicating use of homology to design PCR primers. However, one feature of *dnaN* genes among widely different bacteria is their

10 location in the chromosome. They appear to be near the origin, and immediately adjacent to the *dnaA* gene. *DnaA* genes show good homology among different bacteria and thus we first cloned *dnaA* in order to obtain a DNA probe that is likely near *dnaN*.

Identification of *dnaA* and *dnaN* - The *DnaA* genes of *E. coli* and *B. subtilis* share 58% identity at the amino acid sequence level within the ATP-binding domain (or

15 among the representatives of gram-positive and gram-negative bacteria, evolutionary divergent organisms). Comparison of the predicted amino acid sequences encoded by *dnaA* of *E. coli* and *B. subtilis* revealed two highly conserved regions (Fig. 19). Within each of these regions, a seven amino acid sequence was used to design two oligonucleotide primers for use in the polymerase chain reaction. The DNA

20 oligonucleotides for amplification of *T.th.* genomic DNA were as follows. The upstream 20mer (5'-GTSGTSGTSAAGACSCACTT-3') encodes the following sequence: VLVKTHL. The downstream 21mer (5'-SAGSAGSGCGTTGAASGTGTG-3') encodes the sequence: HTFNALL, on the complementary strand. The amplification reactions contained 10 ng *T.th.* genomic

25 DNA, 0.5 mM of each primer, in a volume of 100 μ l of Vent polymerase reaction mixture containing 10 μ l ThermoPol Buffer, 0.5 mM of each dNTP and 0.5 mM $MgSO_4$. Amplification was performed using the following cycling scheme:

1. 5 cycles of: 95.5°C - 30", 45°C - 30", 75°C - 2'

2. 5 cycles of: 95.5°C - 30", 50°C - 30", 75°C - 2'

3. 30 cycles of: 95.5°C - 30", 52°C - 30", 75°C - 30"

Products were visualized in a 1.5% native agarose gel. A fragment of the expected size of 300 bp was cloned into the SmaI site of pUC19 and sequenced with the

5 CircumVent Thermal Cycle DNA sequencing kit (New England Biolabs).

To obtain a larger section of the *T.th. dnaA* gene, genomic DNA was digested with either HaeII, HindIII, KasI, KpnI, MluI, NcoI, NgoMI, NheI, NsiI, PaeR7I, PstI, SacI, SalI, SpeI, SphI, StuI, or XhoI, followed by Southern analysis in a native agarose gel. The filter was probed with the 300 bp PCR product radiolabeled by random priming.

10 Four different restriction digests showed a single fragment of reasonable size for further cloning. These were, KasI, NgoMI, and StuI which produced fragments of about 3 kb, and NcoI that produced a 2kb fragment. Also, a KpnI digest resulted in two fragments of about 1.5 kb and 10 kb.

Genomic DNA digests using either NgoMI and StuI were used to obtain the *dnaA* gene by inverse PCR (also referred to as circular PCR). In this procedure, 0.1 µg of DNA from each digest was treated separately with T4 DNA ligase in 50 µl of ligation buffer (50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 mg/ml bovine serum albumin) overnight at 20°C. This results in circularizing the genomic DNA fragments. The ligation mixtures were used as substrate in inverse
20 PCR.

DNA oligonucleotides for amplification of recircularized *T.th.* genomic DNA were as follows. The upstream 22mer was 5'-CTCGTTGGTGAAAGTTTCCGTG-3', and the downstream 24mer was 5'-CGTCCAGTTCATCGCCGGAAGGA-3'. The amplification reactions contained 5 ng *T.th.* genomic DNA, 0.5 µM of each primer, in
25 a volume of 100 µl of Taq polymerase reaction mixture containing 10 µl PCR Buffer,

0.5 mM of each dNTP and 2.5 mM MgCl₂. Amplification was performed using the following cycling scheme:

1. 5 cycles of: 95.0°C - 30", 55°C - 30", 72°C - 10'
2. 35 cycles of: 95.5°C - 30", 50°C - 30", 72°C - 8'

- 5 The PCR fragments of the expected length for NgoMI and StuI treated and then ligated chromosomal DNA were digested with either BamHI or Sau3a and cloned into pUC19 : BamHI and pUC19:(BamHI+SmaI) and sequenced with CircumVent Thermal Cycle DNA sequencing kit. The 1.6kb (BamHI+BamH) fragment from the NgoMI PCR product contained a sequence coding for the N-terminal part of *DnaN*,
 10 followed by the gene for enolase. The 1kb (Sau3a+Sau3a) fragment from the same PCR product included the start of *dnaN* gene and sequence characteristic of the origin of replication (i.e. 9mer DnaA-binding site sequences). The 0.6kb (BamHI+BamHI) fragment from the StuI PCR reaction contained starts for *dnaA* and *gidA* genes in inverse orientation to each other. The 0.4 kb (Sau3a+Sau3a) fragment from the same
 15 PCR product contained the 3' end of the *dnaA* gene and DNA sequence characteristic for the origin of replication.

This sequence information provided the beginning and end of both the *dnaA* and the *dnaN* genes. Hence, these genes were easily cloned from this information. Further, the *DnaN* gene was readily cloned and expressed in a pET24-a vector. These steps are
 20 described below.

Cloning and sequence of the *dnaA* gene - The *dnaA* gene was cloned for sequencing in two parts: from the potential start of the gene up to its middle and from the middle up to the end. For the N-terminal part the upstream 27mer
 (5'-TCTGGCAACACGTTCTGGAGCACATCC-3') was 20 bp downstream of the
 25 potential start codon of the gene. The downstream 23mer
 (5'-TGCTGGCGTTCATCTTCAGGATG-3') was approximately from the middle of the *dnaA* gene. For the C-terminal part the upstream 23mer

(5'-CATCCTGAAG.ATGAACGCCAGCA-3') was complementary to the previous primer. The downstream 25mer (5'-AGGTTATCCACAGGGGTCATGTGCA-3') was 20 bp upstream the potential stop codon for the *dnaA* gene. The amplification reactions contained 10 ng *T.th.* genomic DNA, 0.5 μ M of each primer, in a volume of 100 μ l of Vent polymerase reaction mixture containing 10 μ l ThermoPol Buffer, 0.5 mM of each dNTP and 0.5 mM $MgSO_4$. Amplification was performed using the following cycling scheme:

1. 5 cycles of: 95.5°C - 30", 55°C - 30", 75°C - 3'
2. 30 cycles of: 95.5°C - 30", 50°C - 30", 75°C - 2'

Products were visualized in a 1.0% native agarose gel. Fragments of the expected sizes of 750 bp and 650 bp were produced, and were sequenced using CircumVent Thermal Cycle DNA sequencing method (New England Biolabs). The nucleotide and amino acid sequences of *dnaA* and its protein product are shown in Fig. 20. The *DnaA* protein is homologous to the *DnaA* proteins of several other bacteria as shown in Fig. 19.

Cloning and expression of *dnaN* - The full length *dnaN* gene was obtained by PCR from *T.th.* total DNA. DNA oligonucleotides for amplification of *T.th. dnaN* were the following: the upstream 29mer

(5'-GTGTGTCATATGAACATAACGGTTCCCAA-3') consists of an NdeI site

within first 11 nucleotides (underlined), followed by the sequence for the start of the *dnaN* gene; the downstream 29mer

(5'-GCGCGAATTCTCCCTTGTGGAAGGCTTAG-3') consists of an EcoRI site

within the first 10 nucleotides (underlined), followed by the sequence complementary to a section just downstream of the *dnaN* stop codon. The amplification reactions

contained 10 ng *T.th.* genomic DNA, 0.5 μ M of each primer, in a volume of 100 μ l of Vent polymerase reaction mixture containing 10 μ l Thermopol Buffer, 0.5 mM of each dNTP and 0.2 mM $Mg SO_4$. Amplification was performed using the following cycling scheme:

1. 5 cycles of: 95.0°C - 30", 55°C - 30", 75°C - 5',
2. 35 cycles of: 95.5°C - 30", 50°C - 30", 75°C - 4'.

The nucleotide and amino acid sequences of *dnaN* and the β subunit, respectively, are shown in Fig. 21. The *T.th.* β subunit shows limited homology to the β subunit

5 sequences of several other bacteria over its entire length (Fig. 22).

The approximately 1 kb *dnaN* gene was cloned into the pET24-a expression vector using the NdeI and EcoRI restriction sites both in the *dnaN* containing PCR product and in pEt24-a (Fig. 23). Expression of *T.th.* β subunit was obtained under the following conditions: a fresh colony of BL21(DE3) *E.coli* strain was transformed by

10 the pET24-a:*dnaN* plasmid, and then was grown in LB broth containing 50 mg/ml kanamycin at 37°C until the cell density reached 0.4 OD₆₀₀. The cell culture was then induced for *dnaN* expression upon addition of 2 mM IPTG. Cells were harvested after 4 additional hours of growth under 37°C. The induction of the *T.th.* β subunit is shown in Fig. 24.

- 15 Two liters of BL21(DE3)pET*dnaN* cells were grown in LB media containing 50 mg/ml ampicillin at 37°C to an O.D. of 0.8 and then IPTG was added to a concentration of 2 mM. After a further 2 h at 37°C, cells were harvested by centrifugation and stored at -70°C. The following steps were performed at 4°C. Cells were thawed and resuspended in 40 ml of 5 mM Tris-HCl (pH 8.0), 1% sucrose,
- 20 1M NaCl, 5 mM DTT, and 30 mM spermidine. Cells were lysed using a French Pressure cell at 20,000 psi. The lysate was allowed to sit at 4°C for 30 min. and then cell debris was removed by centrifugation (Sorvall SS-34 rotor, 45 min. 18,000 rpm). The supernatant was incubated at 65°C for 20 minutes with occasional stirring. The resulting protein precipitate was removed by centrifugation as described above. The
- 25 supernatant was dialyzed against 4 liters of buffer A containing 50 mM NaCl overnight. The dialyzed supernatant was clarified by centrifugation (35 ml, 150 mg total) and then loaded onto an 8 ml MonoQ column equilibrated in buffer A

containing 50 mM NaCl. The column was washed with 5 column volumes of the same buffer and then eluted with a 120 ml gradient of buffer A plus 50 mM NaCl to buffer A plus 500 mM NaCl. Fractions of 2 ml were collected. Over 50 mg of *T.th.* β was recovered in fractions 5-21.

5

EXAMPLE 13

Alternate synthetic path in absence of clamp loader activity:

As discussed earlier, the Pol III-type enzyme of the present invention is capable of application and use in a variety of contexts, including a method wherein the clamp loader component that is traditionally involved in the initiation of enzyme activity, is
 10 not required. The clamp loader generally functions to increase the efficiency of ring assembly onto circular primed DNA because both the ring and the DNA are circles and one must be broken transiently for them to become interlocked rings. In such a reaction, the clamp loader increases the efficiency of opening the ring.

The procedure described below illustrates the instance where the clamp loader need
 15 not be present. For example, the β clamp can be assembled onto DNA in the absence of the clamp loader. Particularly, the bulk of primed templates in PCR reactions are linear ssDNA fragments that are primed at the ends. On linear primed DNA, the ring need not open at all. Instead, the ring can simply thread onto the end of the linear primed template (Bauer and Burgers, 1988; Tan et. al, 1986; O'Day et. al., 1992;
 20 Burgers and Yoder, 1993). Hence, on linear primed templates, such as those generated in PCR, the beta clamp can simply slide over the DNA end. After the ring slides onto the end, the DNA polymerase can associate with the ring for enhanced DNA synthesis.

Such "end assembly" is common among Pol III-type enzymes and has been
 25 demonstrated in the yeast and human systems. Rings assembling onto linear DNA for use by their respective DNA polymerases are shown in the following example

The bulk of the primed templates in PCR reactions are linear ssDNA fragments that are primed at their ends. However, these end primed linear fragments are not

10 assembly in the absence of a clamp loader.

20 removal of denaturant) leading to rings assembling around the DNA.

synthesized by Oligos etc. The template was prepared using 145 μ l of 5.2 mM (as

nucleotide) polydA and 22 μ ; of 1.75 mM (as nucleotide) oligodT. The mixture was incubated in a final volume of 2100 μ ; T.E. buffer (ratio as nucleotide was 21:1 polydA to oligodT). The mixture was heated to boiling in a 1 ml Eppendorf tube, then removed and allowed to cool to room temperature. Assays were performed in a final volume of 25 μ l 20 mM Tris-Cl (pH 7.5), 8 mM MgCl₂, 5 mM DTT, 0.5 mM EDTA, 40 mg/ml BSA, 4% glycerol, containing 20 μ M [α -³²P]dTTP, 0.1 μ g polydA-oligodT, 25 ng Pol III and, where present, 5 μ g of β subunit. Proteins were added to the reaction on ice, then shifted to 37°C for 5 min. DNA synthesis was quantitated using DE81 paper as described (Rowen and Kornberg, 1979).

- 10 In the linear template assay, no ATP or dATP is provided and therefore, a clamp loader, even if present, is not active. Thus, the clamp (e.g. β) can only stimulate the DNA polymerase provided the clamp threads onto the DNA (see diagram in Fig. 25). Hence, threading of the clamp is shown by a stimulation of the DNA polymerase. In lane 1 of Fig. 25A, the DNA polymerase is incubated with the the linear DNA in the absence of the clamp, and lane 2 shows the result of adding the clamp. The results show that the clamp is able to thread onto the DNA ends and stimulate the DNA polymerase in the absence of ATP and thus, in the absence of clamp loading as well.

- This clamp loader independent assay is performed in the human system in Fig. 25B. The assay reaction (25 μ l) contains 50 mM Tris-HCl (pH=7.8), 8 mM MgCl₂, 1 mM DTT, 1 mM creatine phosphate, 40 μ g/ml bovine serum albumin, 0.55 μ g human SSB, 100 ng PCNA (where present), 7 units DNA polymerase delta (1 unit incorporates 1 pmol dTMP in 60 min.), 40 mM [α -³²P]dTTP and 0.1 μ g polydA-oligodT. Proteins were added to the reaction on ice, then shifted to 37°C for 60 min. DNA synthesis was quantitated using DE81 paper as described (Rowen and Kornberg, 1979). In lane 3, (Fig. 25) the DNA polymerase δ is incubated with the linear DNA in the absence of the clamp, and lane 4 shows the result of adding the PCNA clamp. The results demonstrate that the clamp is able to thread onto the DNA

ends and stimulate the DNA polymerase in the absence of ATP and thus, the absence of clamp loading.

This clamp loader independent assay is performed in the *T.th.* system in Fig. 25C. The assay reaction is exactly as described above for use of the *E. coli* Pol III and beta system except the temperature is 60°C and here the Pol III is HEP.P1 *T.th.* Pol III (0.5 µl, providing 0.1 units where one unit is equal to 1 pmol of dTTP incorporated in 1 minute under these conditions and in the absence of beta), and the beta subunit is 7 µg *T.th.* β (from the MonoQ column). Proteins were added to the reaction on ice, then shifted to 37°C for 60 min. DNA synthesis was quantitated using DE81 paper as described (Rowen and Kornberg, 1979). In lane 3 (Fig. 25C), the *T.Th.* Pol III is incubated with the linear DNA in the absence of the clamp, and lane 4 shows the result of adding the *T.th.* β clamp. The results demonstrate that the clamp is able to thread onto the DNA ends and stimulate the DNA polymerase in the absence of clamp loader activity.

EXAMPLE 14

Use of *T.th.* Pol III in long chain primer extension

A characteristic of Pol III-type enzymes is their ability to extend a single primer for several kilobases around a long (e.g. 7 kb) circular single stranded DNA genome of a bacteriophage. This reaction uses the circular β clamp protein. For the circular β to be assembled onto a circular DNA genome, the circular β must be opened, positioned around the DNA, then closed. This assembly of the circular beta around DNA requires the action of the clamp loader, which uses ATP to open and close the ring around DNA. In this example we use as a template the 7.2 kb circular single strand DNA genome of bacteriophage M13mp18. This template was primed with a single DNA 57mer oligonucleotide and the Pol III enzyme was tested for conversion of this template to a double strand circular form (RFII). The reaction was supplemented with recombinant *T.th.* β produced in *E. coli*. This assay is summarized in the scheme at

the top of Fig. 26. M13mp18 ssDNA was phenol extracted from phage purified as described (Turner and O'Donnell, 1995). M13mp18 ssDNA was primed with a 57mer DNA oligomer synthesized by Oligos etc. The replication assays contained 73 ng singly primed M13mp18 ssDNA and 100 ng *T.th.* β subunit in a 25 μ l reaction

5 containing 20 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 40 μ g/ml BSA, 0.1 mM EDTA, 4% glycerol, 0.5 mM ATP, 60 μ M each of dCTP, dGTP, dATP and 20 μ M α -³²P-TTP (specific activity 2,000-4,000 cpm/pmol). Either *T.th.* Pol III from the Heparin, peak 1 (HEP.P1; 5 μ l, 0.21 units where 1 unit equals 1 pmol nucleotide incorporated in 1 min.) or a non-Pol III from the Heparin peak 2 (HEP.P2; 5 μ l, 2.6 units) were added to

10 the reaction. Reactions were shifted to 60°C for 5 min., and then DNA synthesis was quenched upon adding 25 μ l of 1% SDS, 40 mM EDTA. One half of the reaction was analyzed in a 0.8% native agarose gel, and the other half was quantitated using DE81 paper as described (Studwell and O'Donnell, 1990).

The results of the assay are shown in Fig. 26. Lane 1 is the result obtained using the

15 *T.th.* Pol III (HEP.P1) which was capable of extending the primer around the ssDNA circle to form RFII. Lane 2 shows the result of using the non-Pol III (HEP.P2) which was not capable of this extension and produced only incomplete DNA products (the result shown included 0.8 μ g *E. coli* SSB which did not increase the chain length of the product. In the absence of SSB, the same product was observed, although the

20 band contained more counts. The greater amount of total synthesis observed in lane 2 is due to the build up of immature products in a small region of the gel. The presence of immature products in lane 1 is likely due to a contaminating polymerase in the preparation that can not convert the single primer to the full length RFII form.

Alternatively, the presence of incomplete products in lane 1 (Pol III type enzyme) is

25 due to secondary structure in the DNA which causes the Pol III to pause. In this case it may be presumed that performing the reaction at higher temperature could remove the secondary structure barrier. Alternatively, SSB (single strand binding protein) could be added to the assay (although *T.th.* SSB would be needed since addition of *E. coli* SSB was tried and did not alter the quality of the product profile). Generally, SSB

is needed to remove secondary structure elements from ssDNA at 37°C for complete extension of primers by mesophilic Pol III-type enzymes.

The assay described above was performed at 60°C. The *T.th.* Pol III HEP.P1 gained activity as the temperature was increased from 37°C to 60°C, as expected for an enzyme from a thermophilic source. The *E. coli* Pol III lost activity at 60°C compared to 37°C, as expected for an enzyme from a mesophilic source.

The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. The documents should be considered as incorporated by reference in their entirety.

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[illegible]

(2) INFORMATION FOR SEQ ID NO:6:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

WHAT IS CLAIMED IS:

1. A DNA Polymerase III-type enzyme found in a thermophilic bacterium which exhibits the following characteristics:
- the ability to extend a primer over a long stretch of ssDNA at elevated temperature;
 - the ability to be stimulated by a cognate sliding clamp of the type that is assembled on DNA by a 'clamp' loader (e.g. γ complex);
 - contains associated clamp loading sub-units that show DNA stimulated ATPase activity at elevated temperature and/or ionic strength; and
 - an accessory protein with DNA polymerase-associated 3'-5' exonuclease activity.
2. The DNA Polymerase III-type enzyme according to Claim 1 which is isolated from a thermophilic bacterium selected from *Thermus* and *Thermatoga* species.
3. The DNA Polymerase III-type enzyme according to Claim 2, wherein the thermophilic bacterium comprises a member of the *Thermus* species.
4. The DNA Polymerase III-type enzyme according to Claim 3, wherein the thermophilic bacterium comprises *Thermus thermophilus*.
5. The DNA Polymerase III-type enzyme according to Claim 1, which comprises at least one of the following:
- A. a γ subunit having an amino acid sequence selected from the formula set forth in SEQ ID NOS:4 and 5;
 - B. a τ subunit having an amino acid sequence corresponding to the formula set forth in SEQ ID NO:2;
 - C. a ϵ subunit having an amino acid sequence corresponding to the formula set forth in SEQ ID NO:95;

9 D. a α subunit comprising an amino acid sequence corresponding to the
10 formula set forth in SEQ ID NO:87;

11 E. a β subunit having an amino acid sequence corresponding to the
12 formula set forth in SEQ ID NO:107; and

13 variants, including allelic variants, muteins, analogs and fragments of any of
14 subparts (A) through (E), and combinations thereof, capable of functioning in DNA
15 amplification and sequencing.

1 6. The DNA Polymerase III-type enzyme of Claim 1, which includes a γ sub-unit
2 which exhibits a frameshift as great as -2.

1 7. An isolated polynucleotide encoding a τ subunit of a *Thermus thermophilus*
2 DNA polymerase III-type enzyme, wherein said τ subunit has a molecular weight of
3 about 58,000 daltons as determined by SDS-PAGE under non-reducing conditions.

1 8. An isolated polynucleotide according to Claim 7, wherein said amino acid
2 residue sequence is represented by the formula shown in SEQ ID NO:2. analogs
3 thereof, muteins thereof, alleles thereof, and active fragments thereof.

1 9. An isolated polynucleotide according to Claim 7, wherein the
2 polynucleotide sequence is the polynucleotide sequence of positions 132 to
3 1713 of SEQ ID NO:1, conserved variants thereof, analogs thereof, active fragments
4 thereof, and combinations thereof.

1 10. An isolated polynucleotide according to Claim 7, wherein the polynucleotide
2 is the polynucleotide sequence of positions 1 to 2027 of SEQ ID NO:1, conserved
3 variants thereof, analogs thereof, active fragments thereof, and combinations thereof.

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1 12. The isolated nucleic acid molecule according to Claim 11, wherein said
2 nucleic acid molecule comprises *dnaX*.

1 13. The isolated nucleic acid molecule according to Claim 11, wherein said
2 nucleic acid molecule comprises *dnaQ*.

1 14. An isolated nucleic acid molecule associated with a DNA polymerase III-type
2 enzyme found in a thermophilic bacterium. wherein said nucleic acid molecule
3 comprises *dnaA*.

1 15. The isolated nucleic acid molecule according to Claim 11, wherein said
2 nucleic acid molecule comprises *dnaV*.

1 16. A subunit of a DNA polymerase III-type enzyme found in a thermophilic
2 bacterium, which subunit has an amino acid sequence selected from the group
3 consisting of SEQ ID NO:4; SEQ ID NO:5; SEQ ID NO:2; SEQ ID NO:95; SEQ ID
4 NO:87; SEQ ID NO:107; muteins thereof; alleles thereof; analogs thereof; active
5 fragments thereof; and combinations thereof.

- 1 17. The subunit of Claim 16, wherein said subunit has an amino acid sequence
2 selected from SEQ ID NO:4 and SEQ ID NO:5, and comprises the γ subunit of a
3 *Thermus thermophilus* DNA polymerase III-type enzyme.
- 1 18. The subunit of Claim 16, wherein said subunit has an amino acid sequence set
2 forth in SEQ ID NO:2, and comprises the τ subunit of a *Thermus thermophilus* DNA
3 polymerase III-type enzyme.
- 1 19. The subunit of Claim 16, wherein said subunit has an amino acid sequence set
2 forth in SEQ ID NO:95, and comprises the ϵ subunit of a *Thermus thermophilus* DNA
3 polymerase III-type enzyme.
- 1 20. The subunit of Claim 16, wherein said subunit has an amino acid sequence set
2 forth in SEQ ID NO:87, and comprises the α subunit of a *Thermus thermophilus* DNA
3 polymerase III-type enzyme.
- 1 21. The subunit of Claim 16, wherein said subunit has an amino acid sequence set
2 forth in SEQ ID NO:107, and comprises the β subunit of a *Thermus thermophilus*
3 DNA polymerase III-type enzyme.
- 1 22. A vector comprising an isolated nucleic acid molecule taken from Claim 11.
- 1 23. A vector comprising the isolated nucleic acid molecule of Claim 12.
- 1 24. A vector comprising the isolated nucleic acid molecule of Claim 13.
- 1 25. A vector comprising the isolated nucleic acid molecule of Claim 14.
- 1 26. A vector comprising the isolated nucleic acid molecule of Claim 15.

- 1 27. A vector selected from pET*dnaX* and pET*dnaN*.
- 1 28. A host cell comprising at least one of the vectors of Claim 22.
- 1 29. The host cell according to Claim 28, wherein the host cell is a prokaryotic cell.
- 1 30. A host cell comprising a vector according to Claim 23.
- 1 31. A host cell comprising a vector according to Claim 24.
- 1 32. A host cell comprising a vector according to Claim 25.
- 1 33. A host cell comprising a vector according to Claim 26.
- 1 34. The host cell according to Claim 30, wherein the host cell is a
2 prokaryotic cell.
- 1 35. The host cell according to Claim 31, wherein the host cell is a
2 prokaryotic cell.
- 1 36. The host cell according to Claim 32, wherein the host cell is a
2 prokaryotic cell.
- 1 37. The host cell according to Claim 33, wherein the host cell is a
2 prokaryotic cell.
- 1 38. An isolated DNA which codes for a recombinant DNA polymerase III-type
2 enzyme, or subunit thereof, from a thermophilic bacterium, consisting essentially of a
3 DNA fragment which hybridizes in a Southern blot to an isolated DNA fragment
4 selected from the group consisting of the DNA fragments defined in SEQ ID NO:6

5 and the DNA fragments defined in SEQ ID NO:8, wherein hybridization is conducted
6 under the following conditions:

7 a) hybridization: 1% crystalline BSA (fraction V) (Sigma), 1 mM EDTA,
8 0.5 M NaHPO₄ (pH 7.2), 7% SDS at 65°C for 12 hours and;

9 b) wash: 5 x 20 minutes with wash buffer consisting of 0.5% BSA,
10 fraction V), 1mM Na₂EDTA, 40 mM NaHPO₄ (pH 7.2), and 5% SDS.

1 39. A cloning vector comprising the isolated DNA of Claim 38.

1 40. A host cell transformed by the vector of Claim 39.

1 41. A method for producing a recombinant thermostable DNA polymerase III-type
2 enzyme, or subunit thereof, from a thermophilic bacterium comprising culturing a host
3 cell transformed with the vector of Claim 39 under conditions suitable for the
4 expression of said DNA polymerase III-type enzyme or said subunit.

1 42. A DNA probe which hybridizes to the DNA sequence coding for the
2 thermostable DNA polymerase III-type enzyme, or subunit thereof, of Claim 38,
3 wherein the DNA probe is selected from the group consisting of SEQ ID NO:6 and
4 SEQ ID NO:8.

1 43. A method for isolating a target DNA fragment consisting essentially of
2 a DNA coding for a thermostable DNA polymerase III-type enzyme, or subunit
3 thereof, from a thermophilic bacterium comprising the steps of:
4 (a) forming a genomic library from the bacterium;
5 (b) transforming or transfecting an appropriate host cell with the library of step
6 (a);
7 (c) contacting DNA from the transformed or transfected host cell with a DNA
8 probe which hybridizes to a DNA fragment selected from the group consisting of the

9 DNA fragments defined in SEQ ID NO:6 and the DNA fragments defined in SEQ ID
10 NO:8, wherein hybridization is conducted under the following conditions:

11 i) hybridization: 1% crystalline BSA (fraction V) (Sigma). 1 mM EDTA.
12 0.5 M NaHPO₄ (pH 7.2). 7% SDS at 65°C for 12 hours and;

13 ii) wash: 5 x 20 minutes with wash buffer consisting of 0.5% BSA,
14 fraction V), 1mM Na₂EDTA. 40 mM NaHPO₄ (pH 7.2), and 5% SDS;

15 (d) assaying the transformed or transfected cell of step (c) which hybridizes to
16 the DNA probe for DNA polymerase III-type activity; and

17 (e) isolating a target DNA fragment which codes for the thermostable DNA
18 polymerase III-type enzyme or subunit thereof.

1 44. An isolated DNA molecule encoding a protein subunit of DNA polymerase
2 III-type enzyme from a thermophilic bacterium wherein the subunit group is selected
3 from the group consisting of τ , γ at a -1 frameshift. γ at a -2 frameshift, ϵ , α , and β .

1 45. The isolated DNA molecule according to Claim 44, wherein the subunit is τ
2 and has a molecular weight of 58 kD.

1 46. The isolated DNA molecule according to Claim 45, wherein the protein has an
2 amino acid sequence corresponding to SEQ ID NO:2.

1 47. The isolated DNA molecule according to Claim 45, wherein the DNA
2 molecule has a nucleotide sequence corresponding to SEQ ID NO:3.

1 48. The isolated DNA molecule according to Claim 44, wherein the subunit is γ at
2 a -1 frameshift, and has a molecular weight of 50.8 kD.

1 49. The isolated DNA molecule according to Claim 46, wherein the protein has an
2 amino acid sequence corresponding to SEQ ID NO:4.

- 1 50. The isolated DNA molecule according to Claim 44, wherein the subunit is γ at
2 a -2 frameshift, and has a molecular weight of 49.8 kD.
- 1 51. The isolated DNA molecule according to Claim 47, wherein the protein has an
2 amino acid sequence corresponding to SEQ ID NO:5.
- 1 52. An expression system comprising an isolated nucleic acid molecule according
2 to Claim 11.
- 1 53. The expression system according to Claim 52, wherein the protein corresponds
2 to τ and has an amino acid sequence corresponding to SEQ ID NO:2.
- 1 54. The expression system according to Claim 53, wherein the DNA molecule has
2 a nucleotide sequence corresponding to SEQ ID NO:3.
- 1 55. The expression system according to Claim 52, wherein the protein corresponds
2 to the ϵ subunit and has an amino acid sequence corresponding to SEQ ID NO:95.
- 1 56. The expression system according to Claim 55, wherein said subunit has a
2 nucleotide sequence corresponding to SEQ ID NO:94.
- 1 57. The expression system according to Claim 52, wherein the protein corresponds
2 to the α subunit and has an amino acid sequence corresponding to SEQ ID NO:87.
- 1 58. The expression system according to Claim 57, wherein said subunit has a
2 nucleotide sequence corresponding to SEQ ID NO:86.
- 1 59. The expression system according to Claim 52, wherein the protein corresponds
2 to the β subunit and has an amino acid sequence corresponding to SEQ ID NO:107.

1 60. The expression system according to Claim 59, wherein said subunit has a
2 nucleotide sequence corresponding to SEQ ID NO:106.

1 61. A host cell transformed with a heterologous nucleic acid molecule according
2 to Claim 11.

1 62. The host cell according to Claim 61, wherein the protein has an amino acid
2 sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID
3 NO:5 SEQ ID NO:87, SEQ ID NO:95, and SEQ ID NO:107.

1 63. The host cell according to Claim 61, wherein the nucleic acid molecule has a
2 nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID
3 NO:3, SEQ ID NO:86, SEQ ID NO:94, and SEQ ID NO:106.

1 64. A DNA probe which hybridizes to the DNA sequence coding for the
2 thermostable DNA polymerase III-type enzyme of Claim 1, wherein the DNA probe is
3 selected from the group consisting of the oligonucleotide defined in SEQ ID NO:6;
4 the oligonucleotide defined in SEQ ID NO:8; the oligonucleotide defined in SEQ ID
5 NO:10; the oligonucleotide defined in SEQ ID NO:11; the oligonucleotide defined in
6 SEQ ID NO:12; the oligonucleotide defined in SEQ ID NO:13; the oligonucleotide
7 defined in SEQ ID NO:14; the oligonucleotide defined in SEQ ID NO:15, and the
8 oligonucleotide defined in SEQ ID NO:16.

1 65. A method for isolating a target DNA fragment consisting essentially of
2 a DNA coding for a thermostable DNA polymerase III-type enzyme, or subunit
3 thereof, from a thermophilic bacterium comprising the steps of:
4 (a) forming a genomic library from the bacterium;
5 (b) transforming or transfecting an appropriate host cell with the library of step
6 (a);

16 (e) isolating a target DNA fragment which codes for the thermostable DNA
17 polymerase III-type enzyme or subunit thereof.

1 66. A method for amplifying a nucleic acid molecule, said method comprising
2 contacting said nucleic acid molecule with a composition comprising the DNA
3 polymerase III-type enzyme, or subunit thereof, of Claim 1.

1 67. A DNA molecule amplified by the method of Claim 66.

1 68. A method of preparing a recombinant vector comprising inserting a nucleic
2 acid molecule taken from Claim 11 into a vector.

1 69. The method of Claim 68, wherein said vector is an expression vector.

1 70. A recombinant vector made according to the method of Claim 68.

1 71. A method of making a recombinant host cell, comprising inserting the nucleic
2 acid molecule of Claim 11 into a host cell.

1 72. The method of Claim 71, wherein said host cell is a bacterial cell, a yeast cell
2 or an animal cell.

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As below named inventors, we hereby declare that:

Our residence, post office address and citizenship are as stated below under our names.

We believe that we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled

ENZYME DERIVED FROM THERMOPHILIC ORGANISMS THAT FUNCTIONS AS A CHROMOSOMAL REPLICASE, AND PREPARATION AND USES THEREOF

the Specification of which

☐ is attached hereto
☒ was filed on April 8, 1998
as Application Serial No. 09/057,416
and was amended on _____ (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any provisional application filed in the United States in accordance with 35 U.S.C. §1.119(e), or any application for patent that has been converted to a Provisional Application within one (1) year of its filing date, or any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

<u>APPLICATION</u>	<u>PRIOR FILED APPLICATION(S)</u>	<u>PRIORITY</u>
<u>NUMBER</u>	<u>COUNTRY (DAY/MONTH/YEAR FILED)</u>	<u>CLAIMED</u>
60/043,202	U.S. April 8, 1997	YES

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in any prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a), which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

**STATUS - PATENTED, PENDING,
ABANDONED**

- 2 -

SIGNATURE OF INVENTOR *J. Sullivan*
DATE 5/26/98

SIGNATURE OF INVENTOR Michael E. O'Donnell
DATE 6/1/98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S) : Olga Yurieva *et al*
Serial No. : Unassigned
Filed : Herewith
FOR : ENZYME DERIVED FROM THERMOPHILIC
ORGANISMS THAT FUNCTIONS AS A CHROMOSOMAL
REPLICASE, AND PREPARATION AND USES THEREOF

ASSOCIATE POWER OF ATTORNEY

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

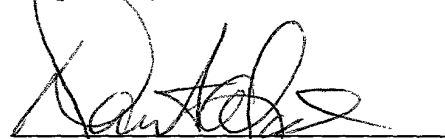
Dear Sir:

Please recognize the following attorneys as associate attorneys in the above-identified
Application:

Allan H. Fried	Registration No. 31,253
Michael A. Yamin	Registration No. 44,414

Please continue to forward all further communications to the undersigned.

Respectfully submitted,



David A. Jackson
Attorney for Applicant
Registration No. 26,742

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DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As below named inventors, we hereby declare that:

Our residence, post office address and citizenship are as stated below under our names.

We believe that we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled

ENZYME DERIVED FROM THERMOPHILIC ORGANISMS THAT FUNCTIONS AS A CHROMOSOMAL REPLICASE, AND PREPARATION AND USES THEREOF

the Specification of which

[] is attached hereto
[X] was filed on April 8, 1998
as Application Serial No. 09/057,416
and was amended on _____ (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any provisional application filed in the United States in accordance with 35 U.S.C. §1.119(e), or any application for patent that has been converted to a Provisional Application within one (1) year of its filing date, or any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

<u>APPLICATION</u> <u>NUMBER</u>	<u>COUNTRY</u>	<u>PRIOR FILED APPLICATION(S)</u> <u>(DAY/MONTH/YEAR FILED)</u>	<u>PRIORITY</u> <u>CLAIMED</u>
60/043,202	U.S.	April 8, 1997	YES

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in any prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.55(a), which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION NO. _____	FILING DATE (DAY/MONTH/YEAR) _____	STATUS - PATENTED, PENDING, ABANDONED _____
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We hereby appoint as our attorneys or agents the following persons: Jack Matalon, (Attorney, Registration No. 22,441); Stefan J. Klauber (Attorney, Registration No. 22,604); David A. Jackson (Attorney, Registration No. 26,742); Michael D. Davis (Attorney, Registration No. 39,161); William C. Coppola (Attorney, Registration No. P41,6867); Mark S. Cohen (Attorney, Registration No. P42,425); and Christine E. Dietzel (Agent, Registration No. 37,309), said attorneys or agents with full power of substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Please address all correspondence regarding this application to:

DAVID A. JACKSON, ESQ.
KLAUBER & JACKSON
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Direct all telephone calls to David A. Jackson at (201) 487-5800.

We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF FIRST JOINT INVENTOR: OLGA YURIEVA

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US

FULL POST OFFICE ADDRESS: 500 E. 63rd Street, Apt. 5E
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SIGNATURE OF INVENTOR 

DATE 05/13/98

FULL NAME OF SECOND JOINT INVENTOR: JOHN KURIYAN

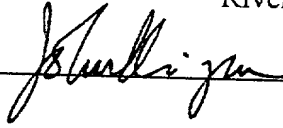
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SIGNATURE OF INVENTOR

DATE 5/26/98



FULL NAME OF THIRD JOINT INVENTOR: MICHAEL E. O'DONNELL

COUNTRY OF CITIZENSHIP: United States

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SIGNATURE OF INVENTOR

DATE

6/1/98



FULL NAME OF FOURTH JOINT INVENTOR: DAVID JERUZALMI

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SIGNATURE OF INVENTOR

David Jeruzalmi

DATE 5/19/98

008720-2122460

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Yurieva, Olga
Kuriyan, John
O'Donnell, Michael E.
Jeruzalmi, David

(ii) TITLE OF INVENTION: ENZYME DERIVED FROM THERMOPHILIC ORGANISMS
THAT FUNCTIONS AS A CHROMOSOMAL REPLICASE, PREPARATION AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 116

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: David A. Jackson, Esq.
(B) STREET: 411 Hackensack Ave, Continental Plaza, 4th
Floor
(C) CITY: Hackensack
(D) STATE: New Jersey
(E) COUNTRY: USA
(F) ZIP: 07601

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/US
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/143,202
(B) FILING DATE: 08-APR-1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/823,407
(B) FILING DATE: 08-APR-1997
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Jackson Esq., David A.
(B) REGISTRATION NUMBER: 26,742
(C) REFERENCE/DOCKET NUMBER: 600-1-179 PCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 201-487-5800
(B) TELEFAX: 201-343-1684

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2007 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

[illegible]

(i) APPLICANT: Yurieva, Olga
Kuriyan, John
O'Donnell, Michael E.
Jeruzalmi, David

(ii) TITLE OF INVENTION: ENZYME DERIVED FROM THERMOPHILIC ORGANISMS THAT FUNCTIONS AS A CHROMOSOMAL REPLICASE, PREPARATION AND USES THEREOF

(iv) CORRESPONDENCE ADDRESS:
 (A) ADDRESSEE: David A. Jackson, Esq.
 (B) STREET: 411 Hackensack Ave, Continental Plaza, 4th
 Floor
 (C) CITY: Hackensack
 (D) STATE: New Jersey
 (E) COUNTRY: USA
 (F) ZIP: 07601

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
 (A) APPLICATION NUMBER: US 08/823,407
 (B) FILING DATE: 08-APR-1997
 (C) CLASSIFICATION:

(ix) TELECOMMUNICATION INFORMATION:
 (A) TELEPHONE: 201-487-5800
 (B) TELEFAX: 201-343-1684

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2007 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

[illegible]

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TCCGGGGGGTG	GGGTTCCCAG	GTAGACCCCG	GCCCCTCCCG	TGAGCCCCTT	TACCCAGGCC	60
GCCACCTCCT	CCAGGGGGGC	CAAGGCGTGC	AAGGAGAGGA	ACGTCCGCAC	CACGCCCTAT	120
ACTAGCCTTG	TGAGCGCCCT	CTACCGCCGC	TTCCGCCCCC	TCACCTTCCA	GGAGGTGGTG	180
GGGCAGGAGC	ACGTGAAGGA	GCCCCTCCTC	AAGGCCATCC	GGGAGGGGAG	GCTCGCCAG	240
GCCTACCTCT	TCTCCGGGCC	CAGGGGCGTG	GGCAAGACCA	CCACGGCGAG	GCTCCTCGCC	300
ATGGCGGTGG	GGTGCCAGGG	GGAAGACCCC	CCTTGCGGGG	TCTGCCCCCA	CTGCCAGGCG	360
GTGCAGAGGG	GCGCCCACCC	GGACGTGGTG	GACATTGACG	CCGCCAGCAA	CAACTCCGTG	420
GAGGACGTGC	GGGAGCTGAG	GGAAAGGATC	CACCTCGCCC	CCCTCTCTGC	CCCCAGGAAG	480
GTCTTCATCC	TGGACGAGGC	CCACATGCTC	TCCAAAAGCG	CCTTCAACGC	CCTCCTCAAG	540
ACCCTGGAGG	AGCCCCCGCC	CCACGTCCCTC	TTCGTCTTCG	CCACCACCGA	GCCCGAGAGG	600
ATGCCCCCCA	CCATCCTCTC	CCGCACCCAG	CACTTCCGCT	TCCGCCGCCT	CACGGAGGAG	660
GAGATCGCCT	TTAAGCTCCG	GCGCATCCTG	GAGGCCGTGG	GGCGGGAGGC	GGAGGAGGAG	720
GCCCTCCTCC	TCCTCGCCCG	CCTGGCGGAC	GGGGCCCTTA	GGGACGCGGA	AAGCCTCCTG	780
GAGCGCTTCC	TCCTCCTGGA	AGGCCCCCTC	ACCCGGAAGG	AGGTGGAGCG	CGCCCTAGGC	840
TCCCCCCCAG	GGACCGGGGT	GGCCGAGATC	GCCGCCTCCC	TCGCGAGGGG	GAAAACGGCG	900
GAGGCCCTGG	GCCTCGCCCG	GCGCCTCTAC	GGGGAAGGGT	ACGCCCCGAG	GAGCCTGGTC	960
TCGGGCCCTT	TGGAGGTGTT	CCGGGAAGGC	CTCTACGCCG	CCTTCGGCCT	CGCGGGAACC	1020
CCCCTTCCCG	CCCCGCCCCA	GGCCCTGATC	GCCGCCATGA	CCGCCCTGGA	CGAGGCCATG	1080
GAGCGCCTCG	CCCGCCGCTC	CGACGCCTTA	AGCCTGGAGG	TGGCCCTCCT	GGAGGCGGGA	1140
AGGGCCCTGG	CCGCCGAGGC	CCTACCCCA	CCCACGGGCG	CTCCTTCCCC	AGAGGTCGGC	1200
CCCAAGCCGG	AAAGCCCCC	GACCCCGGAA	CCCCCAAGGC	CCGAGGAGGC	GCCCCACCTG	1260
CGGGAGCGGT	GGCGGGCCTT	CCTCGAGGCC	CTCAGGCCCA	CCCTACGGGC	CTTCGTGCGG	1320
GAGGCCCCGCC	CGGAGGTCCG	GGAAGGCCAG	CTCTGCCTCG	CTTCCCCGA	GGACAAGGCC	1380
TTCCACTACC	GCAAGGCCTC	GGAACAGAAG	GTGAGGCTCC	TCCCCCTGGC	CCAGGCCCAT	1440
TTCGGGGTGG	AGGAGGTCGT	CCTCGTCCTG	GAGGGAGAAA	AAAAAAGCCT	GAGCCCAAGG	1500
CCCCGCCCCG	CCCCACCTCC	TGAAGCGCCC	GCACCCCCGG	GCCCTCCCGA	GGAGGAGGTA	1560
GAGGCGGAGG	AAGCGGCGGA	GGAGGCCCCG	GAGGAGGCCT	TGAGGCGGGT	GGTCCGCCTC	1620

CTGGGGGGGC GGGTGCTCTG GGTGCGGCGG CCCAGGACCC GGGAGGCGCC GGAGGAGGAA 1680
 CCCCTGAGCC AAGACGAGAT AGGGGGTACT GGTATATAAT GGGGGCATGA CGCGGACCAC 1740
 CGACCTCGGA CAAGAGACCG TGGACAACAT CCTCAAGCGC CTCCGCCGTA TTGAGGGCCA 1800
 GGTGCGGGGG CTCCAGAAGA TGGTGGCCGA GGGCCGCCCC TGCGACGAGG TCCTCACCCA 1860
 GATGACCGCC ACCAAGAAGG CCATGGAGGC GGCGGCCACC CTGATCCTCC ACGAGTTCCT 1920
 GAACGTCTGC GCCGCCGAGG TCTCCGAGGG CAAGGTGAAC CCCAAGAAGC CCGAGGAGAT 1980
 CGCCACCATG CTGAAGAACT TCATCTA 2007

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 529 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Ser	Ala	Leu	Tyr	Arg	Arg	Phe	Arg	Pro	Leu	Thr	Phe	Gln	Glu	Val	1	5	10	15
Val	Gly	Gln	Glu	His	Val	Lys	Glu	Pro	Leu	Leu	Lys	Ala	Ile	Arg	Glu	20	25	30	
Gly	Arg	Leu	Ala	Gln	Ala	Tyr	Leu	Phe	Ser	Gly	Pro	Arg	Gly	Val	Gly	35	40	45	
Lys	Thr	Thr	Thr	Ala	Arg	Leu	Leu	Ala	Met	Ala	Val	Gly	Cys	Gln	Gly	50	55	60	
Glu	Asp	Pro	Pro	Cys	Gly	Val	Cys	Pro	His	Cys	Gln	Ala	Val	Gln	Arg	65	70	75	80
Gly	Ala	His	Pro	Asp	Val	Val	Asp	Ile	Asp	Ala	Ala	Ser	Asn	Asn	Ser	85	90	95	
Val	Glu	Asp	Val	Arg	Glu	Leu	Arg	Glu	Arg	Ile	His	Leu	Ala	Pro	Leu	100	105	110	
Ser	Ala	Pro	Arg	Lys	Val	Phe	Ile	Leu	Asp	Glu	Ala	His	Met	Leu	Ser	115	120	125	
Lys	Ser	Ala	Phe	Asn	Ala	Leu	Leu	Lys	Thr	Leu	Glu	Glu	Pro	Pro	Pro	130	135	140	
His	Val	Leu	Phe	Val	Phe	Ala	Thr	Thr	Glu	Pro	Glu	Arg	Met	Pro	Pro	145	150	155	160

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Thr	Ile	Leu	Ser	Arg	Thr	Gln	His	Phe	Arg	Phe	Arg	Arg	Leu	Thr	Glu	
				165					170						175	
Glu	Glu	Ile	Ala	Phe	Lys	Leu	Arg	Arg	Ile	Leu	Glu	Ala	Val	Gly	Arg	
			180					185					190			
Glu	Ala	Glu	Glu	Glu	Ala	Leu	Leu	Leu	Leu	Ala	Arg	Leu	Ala	Asp	Gly	
		195					200					205				
Ala	Leu	Arg	Asp	Ala	Glu	Ser	Leu	Leu	Glu	Arg	Phe	Leu	Leu	Leu	Glu	
	210					215					220					
Gly	Pro	Leu	Thr	Arg	Lys	Glu	Val	Glu	Arg	Ala	Leu	Gly	Ser	Pro	Pro	
225					230					235					240	
Gly	Thr	Gly	Val	Ala	Glu	Ile	Ala	Ala	Ser	Leu	Ala	Arg	Gly	Lys	Thr	
			245						250					255		
Ala	Glu	Ala	Leu	Gly	Leu	Ala	Arg	Arg	Leu	Tyr	Gly	Glu	Gly	Tyr	Ala	
			260					265						270		
Pro	Arg	Ser	Leu	Val	Ser	Gly	Leu	Leu	Glu	Val	Phe	Arg	Glu	Gly	Leu	
		275					280					285				
Tyr	Ala	Ala	Phe	Gly	Leu	Ala	Gly	Thr	Pro	Leu	Pro	Ala	Pro	Pro	Gln	
	290					295					300					
Ala	Leu	Ile	Ala	Ala	Met	Thr	Ala	Leu	Asp	Glu	Ala	Met	Glu	Arg	Leu	
305					310					315					320	
Ala	Arg	Arg	Ser	Asp	Ala	Leu	Ser	Leu	Glu	Val	Ala	Leu	Leu	Glu	Ala	
			325						330					335		
Gly	Arg	Ala	Leu	Ala	Ala	Glu	Ala	Leu	Pro	Gln	Pro	Thr	Gly	Ala	Pro	
			340					345					350			
Ser	Pro	Glu	Val	Gly	Pro	Lys	Pro	Glu	Ser	Pro	Pro	Thr	Pro	Glu	Pro	
		355					360					365				
Pro	Arg	Pro	Glu	Glu	Ala	Pro	Asp	Leu	Arg	Glu	Arg	Trp	Arg	Ala	Phe	
	370					375					380					
Leu	Glu	Ala	Leu	Arg	Pro	Thr	Leu	Arg	Ala	Phe	Val	Arg	Glu	Ala	Arg	
385					390					395					400	
Pro	Glu	Val	Arg	Glu	Gly	Gln	Leu	Cys	Leu	Ala	Phe	Pro	Glu	Asp	Lys	
			405					410						415		
Ala	Phe	His	Tyr	Arg	Lys	Ala	Ser	Glu	Gln	Lys	Val	Arg	Leu	Leu	Pro	
			420					425					430			
Leu	Ala	Gln	Ala	His	Phe	Gly	Val	Glu	Glu	Val	Val	Leu	Val	Leu	Glu	
		435					440					445				
Gly	Glu	Lys	Lys	Ser	Leu	Ser	Pro	Arg	Pro	Arg	Pro	Ala	Pro	Pro	Pro	
	450					455					460					
Glu	Ala	Pro	Ala	Pro	Pro	Gly	Pro	Pro	Glu	Glu	Glu	Val	Glu	Ala	Glu	
465					470					475					480	
Glu	Ala	Ala	Glu	Glu	Ala	Pro	Glu	Glu	Ala	Leu	Arg	Arg	Val	Val	Arg	
			485						490						495	

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GTGAGCGCCC	TCTACCGCCG	CTTCCGCCCC	CTCACCTTCC	AGGAGGTGGT	GGGGCAGGAG	60
CACGTGAAGG	AGCCCCCTCT	CAAGGCCATC	CGGGAGGGGA	GGCTCGCCCA	GGCCTACCTC	120
TTCTCCGGGC	CCAGGGGCGT	GGGCAAGACC	ACCACGGCGA	GGCTCCTCGC	CATGGCGGTG	180
GGGTGCCAGG	GGGAAGACCC	CCCTTGCGGG	GTCTGCCCCC	ACTGCCAGGC	GGTGCAGAGG	240
GGCGCCACCC	CGGACGTGGT	GGACATTGAC	GCCGCCAGCA	ACAACTCCGT	GGAGGACGTG	300
CGGGAGCTGA	GGGAAAGGAT	CCACCTCGCC	CCCCTCTCTG	CCCCCAGGAA	GGTCTTCATC	360
CTGGACGAGG	CCCACATGCT	CTCCAAAAGC	GCCTTCAACG	CCCTCCTCAA	GACCCTGGAG	420
GAGCCCCCGC	CCCACGTCTT	CTTCGTCTTC	GCCACCACCG	AGCCCCGAGAG	GATGCCCCCC	480
ACCATCCTCT	CCCGCACCCA	GCACTTCCGC	TTCCGCCGCC	TCACGGAGGA	GGAGATCGCC	540
TTTAAGCTCC	GGCGCATCCT	GGAGGCCGTG	GGGCGGGAGG	CGGAGGAGGA	GGCCCTCCTC	600
CTCCTCGCCC	GCCTGGCGGA	CGGGGCCCTT	AGGGACGCGG	AAAGCCTCCT	GGAGCGCTTC	660
CTCCTCCTGG	AAGGCCCCCT	CACCCGGAAG	GAGGTGGAGC	GCGCCCTAGG	CTCCCCCCCA	720
GGGACCGGGG	TGGCCGAGAT	CGCCGCCTCC	CTCGCGAGGG	GGAAAACGGC	GGAGGCCCTG	780
GGCCTCGCCC	GGCGCCTCTA	CGGGGAAGGG	TACGCCCCGA	GGAGCCTGGT	CTCGGGCCTT	840
TTGGAGGTGT	TCCGGGAAGG	CCTCTACGCC	GCCTTCGGCC	TCGCGGGAAC	CCCCCTTCCC	900
GCCCCGCCCC	AGGCCCTGAT	CGCCGCCATG	ACCGCCCTGG	ACGAGGCCAT	GGAGCGCCTC	960
GCCCGCCGCT	CCGACGCCTT	AAGCCTGGAG	GTGGCCCTCC	TGGAGGCGGG	AAGGGCCCTG	1020
GCCGCCGAGG	CCCTACCCCA	GCCCACGGGC	GCTCCTTCCC	CAGAGGTCGG	CCCCAAGCCG	1080

GAAAGCCCCC CGACCCCGGA ACCCCCAAGG CCCGAGGAGG CGCCCGACCT GCGGGAGCGG 1140
TGGCGGGCCT TCCTCGAGGC CCTCAGGCCC ACCCTACGGG CCTTCGTGCG GGAGGCCCCG 1200
CCGGAGGTCC GGAAGGCCA GCTCTGCCTC GCTTTCCCCG AGGACAAGGC CTTCCACTAC 1260
CGCAAGGCCT CGGAACAGAA GGTGAGGCTC CTCCCCCTGG CCCAGGCCCA TTTCGGGGTG 1320
GAGGAGGTCTG TCCTCGTCCT GGAGGGAGAA AAAAAAGCC TGAGCCCAAG GCCCCGCCCG 1380
GCCCCACCTC CTGAAGCGCC CGCACCCCG GGCCCTCCCG AGGAGGAGGT AGAGGCGGAG 1440
GAAGCGGCGG AGGAGGCCCC GGAGGAGGCC TTGAGGCGGG TGGTCCGCCT CCTGGGGGGG 1500
CGGGTGCTCT GGGTGCGCG GCCCAGGACC CGGGAGGCGC CGGAGGAGGA ACCCCTGAGC 1560
CAAGACGAGA TAGGGGGTAC TGGTATATAA 1590

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 464 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Ser	Ala	Leu	Tyr	Arg	Arg	Phe	Arg	Pro	Leu	Thr	Phe	Gln	Glu	Val
1				5					10					15	
Val	Gly	Gln	Glu	His	Val	Lys	Glu	Pro	Leu	Leu	Lys	Ala	Ile	Arg	Glu
			20					25					30		
Gly	Arg	Leu	Ala	Gln	Ala	Tyr	Leu	Phe	Ser	Gly	Pro	Arg	Gly	Val	Gly
		35					40					45			
Lys	Thr	Thr	Thr	Ala	Arg	Leu	Leu	Ala	Met	Ala	Val	Gly	Cys	Gln	Gly
	50					55					60				
Glu	Asp	Pro	Pro	Cys	Gly	Val	Cys	Pro	His	Cys	Gln	Ala	Val	Gln	Arg
65				70					75					80	
Gly	Ala	His	Pro	Asp	Val	Val	Asp	Ile	Asp	Ala	Ala	Ser	Asn	Asn	Ser
			85					90					95		
Val	Glu	Asp	Val	Arg	Glu	Leu	Arg	Glu	Arg	Ile	His	Leu	Ala	Pro	Leu
		100					105					110			
Ser	Ala	Pro	Arg	Lys	Val	Phe	Ile	Leu	Asp	Glu	Ala	His	Met	Leu	Ser
	115					120					125				
Lys	Ser	Ala	Phe	Asn	Ala	Leu	Leu	Lys	Thr	Leu	Glu	Glu	Pro	Pro	Pro

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140

His 145	Val	Leu	Phe	Val	Phe 150	Ala	Thr	Thr	Glu	Pro 155	Glu	Arg	Met	Pro	Pro 160
Thr	Ile	Leu	Ser	Arg 165	Thr	Gln	His	Phe	Arg 170	Phe	Arg	Arg	Leu	Thr	Glu
Glu	Glu	Ile	Ala 180	Phe	Lys	Leu	Arg	Arg 185	Ile	Leu	Glu	Ala	Val	Gly	Arg
Glu	Ala	Glu	Glu	Glu	Ala	Leu	Leu	Leu	Leu	Ala	Arg	Leu	Ala	Asp	Gly
Ala	Leu	Arg	Asp	Ala	Glu	Ser 215	Leu	Leu	Glu	Arg	Phe 220	Leu	Leu	Leu	Glu
Gly 225	Pro	Leu	Thr	Arg	Lys 230	Glu	Val	Glu	Arg	Ala 235	Leu	Gly	Ser	Pro	Pro 240
Gly	Thr	Gly	Val	Ala 245	Glu	Ile	Ala	Ala	Ser 250	Leu	Ala	Arg	Gly	Lys	Thr
Ala	Glu	Ala	Leu	Gly 260	Leu	Ala	Arg	Arg 265	Leu	Tyr	Gly	Glu	Gly	Tyr	Ala
Pro	Arg	Ser 275	Leu	Val	Ser	Gly	Leu 280	Leu	Glu	Val	Phe	Arg 285	Glu	Gly	Leu
Tyr	Ala 290	Ala	Phe	Gly	Leu	Ala 295	Gly	Thr	Pro	Leu	Pro 300	Ala	Pro	Pro	Gln
Ala 305	Leu	Ile	Ala	Ala	Met 310	Thr	Ala	Leu	Asp	Glu 315	Ala	Met	Glu	Arg	Leu 320
Ala	Arg	Arg	Ser	Asp 325	Ala	Leu	Ser	Leu	Glu 330	Val	Ala	Leu	Leu	Glu	Ala
Gly	Arg	Ala	Leu 340	Ala	Ala	Glu	Ala	Leu 345	Pro	Gln	Pro	Thr	Gly	Ala	Pro
Ser	Pro	Glu 355	Val	Gly	Pro	Lys	Pro 360	Glu	Ser	Pro	Pro	Thr 365	Pro	Glu	Pro
Pro	Arg 370	Pro	Glu	Glu	Ala	Pro 375	Asp	Leu	Arg	Glu	Arg 380	Trp	Arg	Ala	Phe
Leu 385	Glu	Ala	Leu	Arg	Pro 390	Thr	Leu	Arg	Ala	Phe 395	Val	Arg	Glu	Ala	Arg 400
Pro	Glu	Val	Arg	Glu 405	Gly	Gln	Leu	Cys	Leu 410	Ala	Phe	Pro	Glu	Asp	Lys
Ala	Phe	His 420	Tyr	Arg	Lys	Ala	Ser	Glu 425	Gln	Lys	Val	Arg	Leu 430	Leu	Pro
Leu	Ala	Gln 435	Ala	His	Phe	Gly	Val 440	Glu	Glu	Val	Val	Leu 445	Val	Leu	Glu
Gly	Glu 450	Lys	Lys	Lys	Pro	Glu 455	Pro	Lys	Ala	Pro	Pro 460	Gly	Pro	Thr	Ser

[illegible]

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Gly Thr Gly Val Ala Glu Ile Ala Ala Ser Leu Ala Arg Gly Lys Thr
 245 250 255
 Ala Glu Ala Leu Gly Leu Ala Arg Arg Leu Tyr Gly Glu Gly Tyr Ala
 260 265 270
 Pro Arg Ser Leu Val Ser Gly Leu Leu Glu Val Phe Arg Glu Gly Leu
 275 280 285
 Tyr Ala Ala Phe Gly Leu Ala Gly Thr Pro Leu Pro Ala Pro Pro Gln
 290 295 300
 Ala Leu Ile Ala Ala Met Thr Ala Leu Asp Glu Ala Met Glu Arg Leu
 305 310 315 320
 Ala Arg Arg Ser Asp Ala Leu Ser Leu Glu Val Ala Leu Leu Glu Ala
 325 330 335
 Gly Arg Ala Leu Ala Ala Glu Ala Leu Pro Gln Pro Thr Gly Ala Pro
 340 345 350
 Ser Pro Glu Val Gly Pro Lys Pro Glu Ser Pro Pro Thr Pro Glu Pro
 355 360 365
 Pro Arg Pro Glu Glu Ala Pro Asp Leu Arg Glu Arg Trp Arg Ala Phe
 370 375 380
 Leu Glu Ala Leu Arg Pro Thr Leu Arg Ala Phe Val Arg Glu Ala Arg
 385 390 395 400
 Pro Glu Val Arg Glu Gly Gln Leu Cys Leu Ala Phe Pro Glu Asp Lys
 405 410 415
 Ala Phe His Tyr Arg Lys Ala Ser Glu Gln Lys Val Arg Leu Leu Pro
 420 425 430
 Leu Ala Gln Ala His Phe Gly Val Glu Glu Val Val Leu Val Leu Glu
 435 440 445
 Gly Glu Lys Lys Lys Ala
 450

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "Primer"
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCAAGCTTC ACGCNTACCT NTTCTCCGGN AC

	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2423	2424	2425	2426	2427	2428	2429	2430	2431	2432	2
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(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

His Ala Tyr Leu Phe Ser Gly Thr
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCGAATTCG TGCTCNGGNG GCTCCTCNAG NGTC

34

(2) INFORMATION FOR SEQ ID NO:9:

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Thr Leu Glu Glu Pro Pro Glu His
1 5

DISCUSSION

(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

38

(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

38

(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

28

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer"
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
GCGCGAATTC GCGCTTCGGG AGGTGGG

27

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer"
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
GCGCGAATTC GGGCGCTTCA GGAGGTGGG

29

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 31 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer"
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
GTGGTGCATA TGGTGAGCGC CCTCTACCGC C

31

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 31 base pairs
 (B) TYPE: nucleic acid

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met Ser Tyr Gln Val Leu Ala Arg Lys Trp Arg Pro Gln Thr Phe Ala
1 5 10 15
Asp Val Val Gly Gln Glu His Val Leu Thr Ala Leu Ala Asn Gly Leu
20 25 30
Ser Leu Gly Arg Ile His His Ala Tyr Leu Phe Ser Gly Thr Arg Gly
35 40 45
Val Gly Lys Thr Ser Ile Ala Arg Leu Leu Ala Lys Gly Leu Asn Cys
50 55 60
Glu Thr Gly Ile Thr Ala Thr Pro Cys Gly Val Cys Asp Asn Cys Arg
65 70 75 80
Glu Ile Glu Gln Gly Arg Phe Val Asp Leu Ile Glu Ile Asp Ala Ala
85 90 95
Ser Arg Thr Lys Val Glu Asp Thr Arg Asp Leu Leu Asp Asn Val Gln
100 105 110
Tyr Ala Pro Ala Arg Gly Arg Phe Lys Val Tyr Leu Ile Asp Glu Val
115 120 125
His Met Leu Ser Arg His Ser Phe Asn Ala Leu Leu Lys Thr Leu Glu
130 135 140
Glu Pro Pro Glu His Val Lys Phe Leu Leu Ala Thr Thr Asp Pro Gln
145 150 155 160
Lys Leu Pro Val Thr Ile Leu Ser Arg Cys Leu Gln Phe His Leu Lys
165 170 175
Ala Leu Asp Val
180

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 180 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO

[illegible][illegible]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 294 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Ser Tyr Gln Val Leu Ala Arg Lys Trp Arg Pro Gln Thr Phe Ala

130	135	140
Lys Thr Leu Glu Glu Pro	Pro Pro His Ala Lys	Phe Ile Phe Ala Thr
145	150	155 160
Thr Glu Ile Arg Lys Val	Pro Val Thr Ile Leu Ser Arg Cys Gln Arg	
	165	170 175
Phe Asp Leu Arg Arg Val Glu Pro Asp Val Leu Val Lys His Phe Asp		
	180	185 190
Arg Ile Ser Ala Lys Glu Gly Ala Arg Ile Glu Met Asp Ala Leu Ala		
	195	200 205
Leu Ile Ala Arg Ala Ala Glu Gly Ser Val Arg Asp Gly Leu Ser Leu		
	210	215 220
Leu Asp Gln Ala Ile Val Gln Thr Glu Arg Gly Gln Thr Val Thr Ser		
	225	230 235 240
Thr Val Val Arg Asp Met Leu Gly Leu Ala Asp Arg Ser Gln Thr Ile		
	245	250 255
Ala Leu Tyr Glu His Val Met Ala Gly Lys Thr Lys Asp Ala Leu Glu		
	260	265 270
Gly Phe Arg Ala Leu Trp Gly Phe Gly Ala Asp Pro Ala Val Val Met		
	275	280 285
Leu Asp Val Leu Asp His Cys His Ala Ser Ala Val		
	290	295 300

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 260 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met	His	Gln	Val	Phe	Tyr	Gln	Lys	Tyr	Arg	Pro	Ile	Asn	Phe	Lys	Gln
1				5					10					15	
Thr	Leu	Gly	Gln	Glu	Ser	Ile	Arg	Lys	Ile	Leu	Val	Asn	Ala	Ile	Asn
			20					25					30		
Arg	Asp	Lys	Leu	Pro	Asn	Gly	Tyr	Ile	Phe	Ser	Gly	Glu	Arg	Gly	Thr
		35				40					45				
Gly	Lys	Thr	Thr	Phe	Ala	Lys	Ile	Ile	Ala	Lys	Ala	Ile	Asn	Cys	Leu
	50					55					60				

00642248 001000

Gly Arg Leu Ala Gln Ala Tyr Leu Phe Ser Gly Pro Arg Gly Val Gly
35 40 45
Lys Thr Thr Thr Ala Arg Leu Leu Ala Met Ala Val Gly Cys Gln Gly
50 55 60
Glu Asp Pro Pro Cys Gly Val Cys Pro His Cys Gln Ala Val Gln Arg
65 70 75 80
Gly Ala His Pro Asp Val Val Asp Ile Asp Ala Ala Ser Asn Asn Ser
85 90 95
Val Glu Asp Val Arg Glu Leu Arg Glu Arg Ile His Leu Ala Pro Leu
100 105 110
Ser Ala Pro Arg Lys Val Phe Ile Leu Asp Glu Ala His Met Leu Ser
115 120 125
Lys Ser Ala Phe Asn Ala Leu Leu Lys Thr Leu Glu Glu Pro Pro Pro
130 135 140
His Val Leu Phe Val Phe Ala Thr Thr Glu Pro Glu Arg Met Pro Pro
145 150 155 160
Thr Ile Leu Ser Arg Thr Gln His Phe Arg Phe Arg Arg Leu Thr Glu
165 170 175
Glu Glu Ile Ala Phe Lys Leu Arg Arg Ile Leu Glu Ala Val Gly Arg
180 185 190
Glu Ala Glu Glu Glu Ala Leu Leu Leu Leu Ala Arg Leu Ala Asp Gly
195 200 205
Ala Leu Arg Asp Ala Glu Ser Leu Leu Glu Arg Phe Leu Leu Leu Glu
210 215 220
Gly Pro Leu Thr Arg Lys Glu Val Glu Arg Ala Leu Gly Ser Pro Pro
225 230 235 240
Gly Thr Gly Val Ala Glu Ile Ala Ala Ser Leu Ala Arg Gly Lys Thr
245 250 255
Ala Glu Ala Leu Gly Leu Ala Arg Arg Leu Tyr Gly Glu Gly Tyr Ala
260 265 270
Pro Arg Ser Leu Val Ser Gly Leu Leu Glu Val Phe Arg Glu Gly Leu
275 280 285
Tyr

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 101 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: RNA (genomic)
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GUCCUGGAGG GAGAAAAAAA AAGCCUGAGC CCAAGGCCCC GCCCGGCCCC ACCUCCUGAA 60

GCGCCCGCAC CCCCCGGGCC UCCCGAGGAG GAGGUAGAGG C 101

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Val Leu Glu Gly Glu Lys Lys Ser Leu Ser Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CACGCNTACC TTTTCTCCGG NAC 23

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer"

[illegible]

GTGCTCNGGN GGCTCCTCNT CNGTC

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

```
(ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "PRIMER"
```

(iii) HYPOTHETICAL: NO

GTGGGATCCG TGGTTCTGGA TCTCGATGAA GAA

33

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

```
(ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "PRIMER"
```

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GTGGGATCCA CGGSCTSTCS GAGCAGAAG

29

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

```
(ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "PRIMER"
```

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GCGGGATCCT CAACGAGGAC CTCTCCATCT TCAA

34

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCGGGATCCT TGTCGTCSAG SGTSAGSGCG TCGTA

35

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GGGAAGGACC AGCGCGTACT CCCCCTGCTC CTAGGTGTG

39

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

[illegible]

27

```
(i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 27 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO
```

27

```
(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "PRIMER"

iii) HYPOTHETICAL: NO
```

30

```
(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 23 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "PRIMER"

ii) HYPOTHETICAL: NO
```

23

1. *Chlorophyll a* (Chl *a*) is the primary photosynthetic pigment in most plants and algae. It is a green pigment that absorbs light energy in the blue and red regions of the visible spectrum.

2. *Chlorophyll b* (Chl *b*) is an accessory pigment found in higher plants and green algae. It absorbs light energy in the blue and orange-red regions of the visible spectrum.

3. *Carotenoids* are a group of pigments that include carotenes and xanthophylls. They absorb light energy in the blue and green regions of the visible spectrum.

4. *Xanthophylls* are a group of pigments that include lutein, zeaxanthin, and antheraxanthin. They absorb light energy in the blue and green regions of the visible spectrum.

5. *Lutein* is a common xanthophyll pigment found in many plants. It absorbs light energy in the blue and green regions of the visible spectrum.

6. *Zeaxanthin* is a xanthophyll pigment that is involved in the photoprotection of photosynthesis. It absorbs light energy in the blue and green regions of the visible spectrum.

7. *Antheraxanthin* is a xanthophyll pigment that is involved in the photoprotection of photosynthesis. It absorbs light energy in the blue and green regions of the visible spectrum.

8. *Anthocyanins* are a group of pigments that include cyanidin, delphinidin, and pelargonidin. They absorb light energy in the blue and green regions of the visible spectrum.

9. *Cyanidin* is a common anthocyanin pigment found in many plants. It absorbs light energy in the blue and green regions of the visible spectrum.

10. *Delphinidin* is an anthocyanin pigment that is involved in the photoprotection of photosynthesis. It absorbs light energy in the blue and green regions of the visible spectrum.

11. *Pelargonidin* is an anthocyanin pigment that is involved in the photoprotection of photosynthesis. It absorbs light energy in the blue and green regions of the visible spectrum.

12. *Flavonoids* are a group of pigments that include flavones, flavonols, and flavanones. They absorb light energy in the blue and green regions of the visible spectrum.

13. *Flavones* are a group of pigments that include chrysin, apigenin, and luteolin. They absorb light energy in the blue and green regions of the visible spectrum.

14. *Flavonols* are a group of pigments that include quercetin, kaempferol, and myricetin. They absorb light energy in the blue and green regions of the visible spectrum.

15. *Flavanones* are a group of pigments that include hesperetin, naringenin, and eriodictyol. They absorb light energy in the blue and green regions of the visible spectrum.

16. *Quercetin* is a common flavonol pigment found in many plants. It absorbs light energy in the blue and green regions of the visible spectrum.

17. *Kaempferol* is a flavonol pigment that is involved in the photoprotection of photosynthesis. It absorbs light energy in the blue and green regions of the visible spectrum.

18. *Myricetin* is a flavonol pigment that is involved in the photoprotection of photosynthesis. It absorbs light energy in the blue and green regions of the visible spectrum.

19. *Hesperetin* is a flavanone pigment that is involved in the photoprotection of photosynthesis. It absorbs light energy in the blue and green regions of the visible spectrum.

20. *Naringenin* is a flavanone pigment that is involved in the photoprotection of photosynthesis. It absorbs light energy in the blue and green regions of the visible spectrum.

21. *Eriodictyol* is a flavanone pigment that is involved in the photoprotection of photosynthesis. It absorbs light energy in the blue and green regions of the visible spectrum.

22. *Stilbenes* are a group of pigments that include resveratrol, pterostilbenes, and stilbenes. They absorb light energy in the blue and green regions of the visible spectrum.

23. *Resveratrol* is a common stilbene pigment found in many plants. It absorbs light energy in the blue and green regions of the visible spectrum.

24. *Pterostilbenes* are a group of pigments that include pterostilbenes, pterostilbenes, and pterostilbenes. They absorb light energy in the blue and green regions of the visible spectrum.

25. *Stilbenes* are a group of pigments that include stilbenes, stilbenes, and stilbenes. They absorb light energy in the blue and green regions of the visible spectrum.

```
(ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "PRIMER"
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

19

```
(ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "PRIMER"
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

19

```
(ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "PRIMER"
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

27

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "PRIMER"

- (iii) HYPOTHETICAL: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GAASCCSNNG TCGAASNNGG CGTTGTG

27

- (2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "PRIMER"

- (iii) HYPOTHETICAL: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CGGGGATCCA CCTCAATCAC CTCGTGG

27

- (2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "PRIMER"

- (iii) HYPOTHETICAL: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CGGGGATCCG CCACCTTGCG GCTCCGGGTG

30

- (2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 31 base pairs
 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GCGCTCTAGA CGAGTTCCCA AAGCGTGCGG T

31

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CGCGTCTAGA TCACCTGTAT CCAGA

25

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GCGGCGCATA TGGTGGTGGT CCTGGACCTG GAG

33

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"
(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CGCGTCTAGA TCACCTGTAT CCAGA

25

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"
(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GTSCTS GTSA AGACSCACTT

20

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"
(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

SAGSAGSGCG TTGAASGTGT G

21

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

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27

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(i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 23 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO
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29

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

29

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

Phe Phe Ile Glu Ile Gln Asn His Gly Leu Ser Glu Gln Lys
1 5 10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

INFORMATION FOR SEO ID NO:63:

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

(2) INFORMATION FOR SEQ ID NO:64:

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

(2) INFORMATION FOR SEQ ID NO:65:

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Phe Asn Lys Ser His Ser Ala Ala Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:66:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Val Val Xaa Asp Xaa Glu Thr Thr Gly
1 5

(2) INFORMATION FOR SEQ ID NO:67:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

His Asn Ala Xaa Phe Asp Xaa Gly Phe
1 5

(2) INFORMATION FOR SEQ ID NO:68:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Val Val Xaa Asp Xaa Glu Thr Thr Gly
1 5

(2) INFORMATION FOR SEQ ID NO:69:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids

[illegible]

```
Val Leu Val Lys Thr His Leu
1          5
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(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

His Arg Ala Leu Tyr Asp
1 5

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

His Thr Phe Asn Ala Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2
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Asp Arg Tyr Phe Leu Glu Leu Ile Arg Thr Gly Arg Pro Asp Glu Glu
1 5 10 15
Ser Tyr Leu His Ala Ala Val Glu Leu Ala Glu Ala Arg Gly Leu Pro
20 25 30
Val Val

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

```

Asp His Phe Tyr Leu Glu Leu Ile Arg Thr Gly Arg Ala Asp Glu Glu
1          5          10          15
Ser Tyr Leu His Phe Ala Leu Asp Val Ala Glu Gln Tyr Asp Leu Pro
          20          25          30
Val Val

```

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

Asp His Phe Tyr Leu Ala Leu Ser Arg Thr Gly Arg Pro Asn Glu Glu
1 5 10 15
Arg Tyr Ile Gln Ala Ala Leu Lys Leu Ala Glu Arg Cys Asp Leu Pro
20 25 30
Leu Val

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:

[illegible]

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

Phe Ile Glu Asn Ser Tyr Ile Gln Ile Ala Ser Glu Leu Ser Ile Pro
20 25 30

(2) INFORMATION FOR SEQ ID NO:76:

(ii) MOLECULE TYPE: protein

Asp Asp Phe Tyr Leu Glu Ile Met Arg His Gly Ile Leu Asp Gln Arg
1 5 10 15

Ile Ile

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

Asp Asp Tyr Tyr Leu Glu Ile Gln Asp His Gly Ser Val Glu Asp Arg
1 5 10 15

Figure 1. Schematic representation of the experimental design. The subjects were divided into two groups: the control group (CG) and the experimental group (EG). The CG was divided into two subgroups: the control group (CG) and the control group (CG). The EG was divided into two subgroups: the experimental group (EG) and the experimental group (EG). The CG was divided into two subgroups: the control group (CG) and the control group (CG). The EG was divided into two subgroups: the experimental group (EG) and the experimental group (EG).



(ii) MOLECULE TYPE: protein

Asp Asn Tyr Phe Leu Glu Leu Met Asp His Gly Leu Thr Ile Glu Arg
1 5 10 15

Pro Leu

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 46 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Ile Pro Leu Asp Asp Lys Lys Ser Phe Asp Met Leu Gln Arg Ser Glu
20 25 30

Thr Thr Ala Val Phe Gln Leu Glu Ser Arg Gly Met Lys Asp
35 40 45

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

Asn Pro Arg Leu Lys Lys Ala Gly Lys Pro Pro Val Arg Ile Glu Ala
1 5 10 15
Ile Pro Leu Asp Asp Ala Arg Ser Phe Arg Asn Leu Gln Asp Ala Lys
20 25 30
Thr Thr Ala Val Phe Gln Leu Glu Ser Arg Gly Met Lys Glu
35 40 45

(2) INFORMATION FOR SEQ ID NO:81:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Asn Val Arg Met Val Arg Glu Gly Lys Pro Arg Val Asp Ile Ala Ala
1 5 10 15
Ile Pro Leu Asp Asp Pro Glu Ser Phe Glu Leu Leu Lys Arg Ser Glu
20 25 30
Thr Thr Ala Val Phe Gln Leu Glu Ser Arg Gly Met Lys Asp
35 40 45

(2) INFORMATION FOR SEQ ID NO:82:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

Cys Lys Lys Leu Leu Lys Glu Gln Gly Ile Lys Ile Asp Phe Asp Asp
1 5 10 15
Met Thr Phe Asp Asp Lys Lys Thr Tyr Gln Met Leu Cys Lys Gly Lys
20 25 30
Gly Val Gly Val Phe Gln Phe Glu Ser Ile Gly Met Lys Asp
35 40 45

[illegible]

(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

Leu	Lys	Ile	Ile	Lys	Thr	Gln	His	Lys	Ile	Ser	Val	Asp	Phe	Leu	Ser
1				5					10					15	
Leu	Asp	Met	Asp	Asp	Pro	Lys	Val	Tyr	Lys	Thr	Ile	Gln	Ser	Gly	Asp
			20					25					30		
Thr	Val	Gly	Ile	Phe	Gln	Ile	Glu	Ser	Gly	Met	Phe	Gln			
		35					40					45			

(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

Gln Glu Arg Lys Ala Leu Gln Ile Arg Ala Arg Thr Gly Ser Lys Lys
1 5 10 15

Leu Pro Asp Asp Val Lys Lys Thr His Lys Leu Leu Glu Ala Gly Asp
20 25 30

Leu Glu Gly Ile Phe Gln Leu Glu Ser Gln Gly Met Lys Gln
35 40 45

(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 394 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Met	Gly	Arg	Glu	Leu	Arg	Phe	Ala	His	Leu	His	Gln	His	Thr	Gln	Phe	
1				5					10					15		
Ser	Leu	Leu	Asp	Gly	Ala	Pro	Lys	Leu	Ser	Asp	Leu	Leu	Lys	Trp	Val	
			20					25					30			
Glu	Glu	Thr	Thr	Pro	Glu	Asp	Pro	Ala	Leu	Ala	Met	Thr	Asp	His	Gly	
		35					40					45				
Asn	Leu	Phe	Gly	Ala	Val	Glu	Phe	Tyr	Lys	Lys	Ala	Ala	Glu	Met	Gly	
	50					55					60					
Ile	Glu	Pro	Ile	Leu	Gly	Thr	Arg	Pro	Tyr	Val	Ala	Ala	Glu	Ser	Pro	
65					70					75					80	
Phe	Asp	Arg	Lys	Arg	Gly	Lys	Gly	Leu	Asp	Gly	Gly	Tyr	Phe	His	Leu	
			85					90						95		
Thr	Leu	Leu	Ala	Lys	Asp	Phe	Thr	Gly	Tyr	Gln	Asn	Leu	Val	Arg	Leu	
			100					105						110		
Ala	Ser	Arg	Ala	Tyr	Leu	Glu	Gly	Phe	Tyr	Glu	Lys	Pro	Arg	Ile	Asp	
		115					120					125				
Arg	Glu	Ile	Leu	Arg	Glu	Arg	Arg	Gly	Pro	His	Arg	Leu	Ser	Gly	Cys	
	130					135					140					
Leu	Gly	Ala	Glu	Ile	Pro	Gln	Phe	Ile	Leu	Gln	Asp	Arg	Leu	Asp	Leu	
145					150					155					160	
Phe	Phe	Ile	Glu	Ile	Gln	Asn	His	Gly	Leu	Ser	Glu	Gln	Lys	Ala	Glu	
			165						170					175		
Ala	Arg	Leu	Asn	Glu	Asp	Leu	Ser	Ile	Phe	Lys	Asp	Arg	Phe	Phe	Ile	
			180					185						190		
His	Ile	Gln	Asn	His	Gly	Leu	Pro	Glu	Gln	Lys	Lys	Val	Asn	Glu	Val	
		195					200					205				
Leu	Lys	Glu	Phe	Ala	Arg	Lys	Tyr	Gly	Leu	Gly	Met	Val	Ala	Thr	Asn	
	210					215					220					
Asp	Gly	His	Tyr	Gly	Arg	Lys	Glu	Ala	Arg	Ser	Ala	His	Glu	Val	Phe	
225					230					235					240	
Leu	Ala	Ile	Gln	Ser	Lys	Ser	Thr	Leu	Asp	Asp	Pro	Gly	Ala	Val	Gly	
			245					250						255		

002120-0124350

[illegible]

(2) INFORMATION FOR SEQ ID NO:89:

- (A) LENGTH: 182 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

Ser Tyr Gly Asp Val Gln Val Thr Ala Gln Ala Tyr Leu Arg Leu Leu
 165 170 175

Glu Leu Leu Gly Glu Arg
 180

(2) INFORMATION FOR SEQ ID NO:90:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 201 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

His Gly Ile Lys Met Ile Tyr Gly Met Glu Ala Asn Leu Val Asp Asp
 1 5 10 15
 Gly Val Pro Ile Ala Tyr Asn Ala Ala His Arg Leu Leu Glu Glu Glu
 20 25 30
 Thr Tyr Val Val Phe Asp Val Glu Thr Thr Gly Leu Ser Ala Val Tyr
 35 40 45
 Asp Thr Ile Ile Glu Leu Ala Ala Val Lys Val Lys Gly Gly Glu Ile
 50 55 60
 Ile Asp Lys Phe Glu Ala Phe Ala Asn Pro His Arg Pro Leu Ser Ala
 65 70 75 80
 Thr Ile Ile Glu Leu Thr Gly Ile Thr Asp Asp Met Leu Gln Asp Ala
 85 90 95
 Pro Asp Val Val Asp Val Ile Arg Asp Phe Arg Glu Trp Ile Gly Asp
 100 105 110
 Asp Ile Leu Val Ala His Asn Ala Ser Phe Asp Met Gly Phe Leu Asn
 115 120 125
 Val Ala Tyr Lys Lys Leu Leu Glu Val Glu Lys Ala Lys Asn Pro Val
 130 135 140
 Ile Asp Thr Leu Glu Leu Gly Arg Phe Leu Tyr Pro Glu Phe Lys Asn
 145 150 155 160
 His Arg Leu Asn Thr Leu Cys Lys Lys Phe Asp Ile Glu Leu Thr Gln
 165 170 175
 His His Arg Ala Ile Tyr Asp Thr Glu Ala Thr Ala Tyr Leu Leu Leu
 180 185 190
 Lys Met Leu Lys Asp Ala Ala Glu Lys
 195 200

(2) INFORMATION FOR SEQ ID NO:91:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100			
1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100			
2		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100		
3			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	
4				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
5					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75																								

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

(2) INFORMATION FOR SEQ ID NO:92:

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Ile Asn Arg Phe Glu Thr Leu Val Lys Val Lys Ser Val Pro Asp Tyr
65 70 75 80

Ile Ala Glu Leu Thr Gly Ile Thr Tyr Glu Asp Thr Leu Asn Ala Pro
85 90 95

Ser Ala His Glu Ala Leu Gln Glu Leu Arg Leu Phe Leu Gly Asn Ser
100 105 110

Val Phe Val Ala His Asn Ala Asn Phe Asp Tyr Asn Phe Leu Gly Arg
115 120 125

Tyr Phe Val Glu Lys Leu His Cys Pro Leu Leu Asn Leu Lys Leu Cys
130 135 140

Thr Leu Asp Leu Ser Lys Arg Ala Ile Leu Ser Met Arg Tyr Ser Leu
145 150 155 160

Ser Phe Leu Lys Glu Leu Leu Gly Phe Gly Ile Glu Val Ser His Arg
165 170 175

Ala Tyr Ala Asp Ala Leu Ala Ser Tyr Lys Leu Phe Glu Ile Cys Leu
180 185 190

Leu Asn Leu Pro Ser Tyr Ile Lys Thr
195 200

(2) INFORMATION FOR SEQ ID NO:94:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 630 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

ATGGTGGAGC GGGTGGTGCG GACCCCTTCTG GACGGGAGGT TCCTCCTGGA GGAGGGGGTG 60

GGGCTTTGGG AGTGGCGCTA CCCCTTTCCC CTGGAGGGGG AGGCGGTGGT GGTCTTGAC 120

CTGGAGACCA CGGGGCTTGC CGGCCTGGAC GAGGTGATTG AGGTGGGCCT CCTCCGCCTG 180

GAGGGGGGGA GGCGCCTCCC CTTCCAGAGC CTCGTCCGGC CCCTCCCGCC CGCCGAAGCC 240

CGTTCGTGGA ACCTCACCGG CATCCCCCGG GAGGCCCTGG AGGAGGCCCC CTCCCTGGAG 300

GAGGTTCTGG AGAAGGCCTA CCCCCTCCGC GGCGACGCCA CCTTGGTGAT CCACAACGCC 360

GCCTTTGACC TGGGCTTCCT CCGCCCGGCC TTGGAGGGCC TGGGCTACCG CCTGGAAAAC 420

CCCGTGGTGG ACTCCCTGCG CTTGGCCAGA CGGGGCTTAC CAGGCCTTAG GCGCTACGGC 480

CTGGACGCCC TCTCCGAGGT CCTGGAGCTT CCCCGAAGGA CCTGCCACCG GGCCCTCGAG 540

GACGTGGAGC GCACCCTCGC CGTGGTGCAC GAGGTATACT ATATGCTTAC GTCCGGCCGT 600

(2) INFORMATION FOR SEQ ID NO:95:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 210 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

Met Val Glu Arg Val Val Arg Thr Leu Leu Asp Gly Arg Phe Leu Leu
 1 5 10 15
 Glu Glu Gly Val Gly Leu Trp Glu Trp Arg Tyr Pro Phe Pro Leu Glu
 20 25 30
 Gly Glu Ala Val Val Val Leu Asp Leu Glu Thr Thr Gly Leu Ala Gly
 35 40 45
 Leu Asp Glu Val Ile Glu Val Gly Leu Leu Arg Leu Glu Gly Gly Arg
 50 55 60
 Arg Leu Pro Phe Gln Ser Leu Val Arg Pro Leu Pro Pro Ala Glu Ala
 65 70 75 80
 Arg Ser Trp Asn Leu Thr Gly Ile Pro Arg Glu Ala Leu Glu Glu Ala
 85 90 95
 Pro Ser Leu Glu Glu Val Leu Glu Lys Ala Tyr Pro Leu Arg Gly Asp
 100 105 110
 Ala Thr Leu Val Ile His Asn Ala Ala Phe Asp Leu Gly Phe Leu Arg
 115 120 125
 Pro Ala Leu Glu Gly Leu Gly Tyr Arg Leu Glu Asn Pro Val Val Asp
 130 135 140
 Ser Leu Arg Leu Ala Arg Arg Gly Leu Pro Gly Leu Arg Arg Tyr Gly
 145 150 155 160
 Leu Asp Ala Leu Ser Glu Val Leu Glu Leu Pro Arg Arg Thr Cys His
 165 170 175
 Arg Ala Leu Glu Asp Val Glu Arg Thr Leu Ala Val Val His Glu Val
 180 185 190
 Tyr Tyr Met Leu Thr Ser Gly Arg Pro Arg Thr Leu Trp Glu Leu Gly
 195 200 205
 Arg Glx
 210

(2) INFORMATION FOR SEQ ID NO:96:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 461 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

Met Leu Glu Ala Ser Trp Glu Lys Val Gln Ser Ser Leu Lys Gln Asn
1 5 10 15
Leu Ser Lys Pro Ser Tyr Glu Thr Trp Ile Arg Pro Thr Glu Phe Ser
20 25 30
Gly Phe Lys Asn Gly Glu Leu Thr Leu Ile Ala Pro Asn Ser Phe Ser
35 40 45
Ser Ala Trp Leu Lys Asn Asn Tyr Ser Gln Thr Ile Gln Glu Thr Ala
50 55 60
Glu Glu Ile Phe Gly Glu Pro Val Thr Val His Val Lys Val Lys Ala
65 70 75 80
Asn Ala Glu Ser Ser Asp Glu His Tyr Ser Ser Ala Pro Ile Thr Pro
85 90 95
Pro Leu Glu Ala Ser Pro Gly Ser Val Asp Ser Ser Gly Ser Ser Leu
100 105 110
Arg Leu Ser Lys Lys Thr Leu Pro Leu Leu Asn Leu Arg Tyr Val Phe
115 120 125
Asn Arg Phe Val Val Gly Pro Asn Ser Arg Met Ala His Ala Ala Ala
130 135 140
Met Ala Val Ala Glu Ser Pro Gly Arg Glu Phe Asn Pro Leu Phe Ile
145 150 155 160
Cys Gly Gly Val Gly Leu Gly Lys Thr His Leu Met Gln Ala Ile Gly
165 170 175
His Tyr Arg Leu Glu Ile Asp Pro Gly Ala Lys Val Ser Tyr Val Ser
180 185 190
Thr Glu Thr Phe Thr Asn Asp Leu Ile Leu Ala Ile Arg Gln Asp Arg
195 200 205
Met Gln Ala Phe Arg Asp Arg Tyr Arg Ala Ala Asp Leu Ile Leu Val
210 215 220
Asp Asp Ile Gln Phe Ile Glu Gly Lys Glu Tyr Thr Gln Glu Glu Phe
225 230 235 240
Phe His Thr Phe Asn Ala Leu His Asp Ala Gly Ser Gln Ile Val Leu
245 250 255
Ala Ser Asp Arg Pro Pro Ser Gln Ile Pro Arg Leu Gln Glu Arg Leu
260 265 270
Met Ser Arg Phe Ser Met Gly Leu Ile Ala Asp Val Gln Ala Pro Asp

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65	70								75					80			
Asp	Gly	Leu	Glu	Pro	His	Ser	Leu	Ile	Gly	Gln	Glu	Ser	Ser	Leu	Pro		
				85					90					95			
Met	Glu	Thr	Thr	Pro	Lys	Asn	Ala	Thr	Ala	Leu	Asn	Gly	Lys	Tyr	Thr		
			100					105					110				
Phe	Ser	Arg	Phe	Val	Val	Gly	Pro	Thr	Asn	Arg	Met	Ala	His	Ala	Ala		
		115					120					125					
Ser	Leu	Ala	Val	Ala	Glu	Ser	Pro	Gly	Arg	Glu	Phe	Asn	Pro	Leu	Phe		
	130					135					140						
Leu	Cys	Gly	Gly	Val	Gly	Leu	Gly	Lys	Thr	His	Leu	Met	Gln	Ala	Ile		
145					150					155					160		
Ala	His	Tyr	Arg	Leu	Glu	Met	Tyr	Pro	Asn	Ala	Lys	Val	Tyr	Tyr	Val		
				165					170					175			
Ser	Thr	Glu	Arg	Phe	Thr	Asn	Asp	Leu	Ile	Thr	Ala	Ile	Arg	Gln	Asp		
			180					185					190				
Asn	Met	Glu	Asp	Phe	Arg	Ser	Tyr	Tyr	Arg	Ser	Ala	Asp	Phe	Leu	Leu		
		195					200					205					
Ile	Asp	Asp	Ile	Gln	Phe	Ile	Lys	Gly	Lys	Glu	Tyr	Thr	Gln	Glu	Glu		
	210					215					220						
Phe	Phe	His	Thr	Phe	Asn	Ser	Leu	His	Glu	Ala	Gly	Lys	Gln	Val	Val		
225					230					235					240		
Val	Ala	Ser	Asp	Arg	Ala	Pro	Gln	Arg	Ile	Pro	Gly	Leu	Gln	Asp	Arg		
				245					250					255			
Leu	Ile	Ser	Arg	Phe	Ser	Met	Gly	Leu	Ile	Ala	Asp	Ile	Gln	Val	Pro		
			260					265					270				
Asp	Leu	Glu	Thr	Arg	Met	Ala	Ile	Leu	Gln	Lys	Lys	Ala	Glu	Tyr	Asp		
		275					280					285					
Arg	Ile	Arg	Leu	Pro	Lys	Glu	Val	Ile	Glu	Tyr	Ile	Ala	Ser	His	Tyr		
	290					295					300						
Thr	Ser	Asn	Ile	Arg	Glu	Leu	Glu	Gly	Ala	Leu	Ile	Arg	Ala	Ile	Ala		
305					310					315					320		
Tyr	Thr	Ser	Leu	Ser	Asn	Val	Ala	Met	Thr	Val	Glu	Asn	Ile	Ala	Pro		
				325					330					335			
Val	Leu	Asn	Pro	Pro	Val	Glu	Lys	Val	Ala	Ala	Ala	Pro	Glu	Thr	Ile		
			340					345					350				
Ile	Thr	Ile	Val	Ala	Gln	His	Tyr	Gln	Leu	Lys	Val	Glu	Glu	Leu	Leu		
		355					360					365					
Ser	Asn	Ser	Arg	Arg	Arg	Glu	Val	Ser	Leu	Ala	Arg	Gln	Val	Gly	Met		
	370					375					380						
Tyr	Leu	Met	Arg	Gln	His	Thr	Asp	Leu	Ser	Leu	Pro	Arg	Ile	Gly	Glu		
385					390					395					400		
Ala	Phe	Gly	Gly	Lys	Asp	His	Thr	Thr	Val	Met	Tyr	Ser	Cys	Asp	Lys		

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100					105					110					
Pro	Thr	Thr	Pro	Trp	Pro	His	Gly	Gly	Ala	Val	Ala	Val	Ala	Glu	Ser
		115					120					125			
Pro	Gly	Arg	Ala	Tyr	Asn	Pro	Leu	Phe	Ile	Tyr	Gly	Gly	Arg	Gly	Leu
	130					135					140				
Gly	Lys	Thr	Tyr	Leu	Met	His	Ala	Val	Gly	Pro	Leu	Arg	Ala	Lys	Arg
145					150					155					160
Phe	Pro	His	Met	Arg	Leu	Glu	Tyr	Val	Ser	Thr	Glu	Thr	Phe	Thr	Asn
			165						170					175	
Glu	Leu	Ile	Asn	Arg	Pro	Ser	Ala	Arg	Asp	Arg	Met	Thr	Glu	Phe	Arg
			180					185					190		
Glu	Arg	Tyr	Arg	Ser	Val	Asp	Leu	Leu	Leu	Val	Asp	Asp	Val	Gln	Phe
		195					200					205			
Ile	Ala	Gly	Lys	Glu	Arg	Thr	Gln	Glu	Glu	Phe	Phe	His	Thr	Phe	Asn
	210					215					220				
Ala	Leu	Tyr	Glu	Ala	His	Lys	Gln	Ile	Ile	Leu	Ser	Ser	Asp	Arg	Pro
225						230					235				240
Pro	Lys	Asp	Ile	Leu	Thr	Leu	Glu	Ala	Arg	Leu	Arg	Ser	Arg	Phe	Glu
				245					250					255	
Trp	Gly	Leu	Ile	Thr	Asp	Asn	Pro	Ala	Pro	Asp	Leu	Glu	Thr	Arg	Ile
			260					265					270		
Ala	Ile	Leu	Lys	Met	Asn	Ala	Ser	Ser	Gly	Pro	Glu	Asp	Pro	Glu	Asp
		275					280					285			
Ala	Leu	Glu	Tyr	Ile	Ala	Arg	Gln	Val	Thr	Ser	Asn	Ile	Arg	Glu	Trp
		290				295					300				
Glu	Gly	Ala	Leu	Met	Arg	Ala	Ser	Pro	Phe	Ala	Ser	Leu	Asn	Gly	Val
305					310					315					320
Glu	Leu	Thr	Arg	Ala	Val	Ala	Ala	Lys	Ala	Leu	Arg	His	Leu	Arg	Pro
				325					330					335	
Arg	Glu	Leu	Glu	Ala	Asp	Pro	Leu	Glu	Ile	Ile	Arg	Lys	Ala	Ala	Gly
			340					345					350		
Pro	Val	Arg	Pro	Glu	Thr	Pro	Gly	Gly	Ala	His	Gly	Glu	Arg	Arg	Lys
		355					360					365			
Lys	Glu	Val	Val	Leu	Pro	Arg	Gln	Leu	Ala	Met	Tyr	Leu	Val	Arg	Glu
	370					375					380				
Leu	Thr	Pro	Ala	Ser	Leu	Pro	Glu	Ile	Gly	Gln	Leu	Phe	Gly	Gly	Arg
385					390					395					400
Asp	His	Thr	Thr	Val	Arg	Tyr	Ala	Ile	Gln	Lys	Val	Gln	Glu	Leu	Ala
				405					410					415	
Gly	Lys	Pro	Asp	Arg	Glu	Val	Gln	Gly	Leu	Leu	Arg	Thr	Leu	Arg	Glu
			420					425					430		
Ala	Cys	Thr	Asp	Pro	Val	Asp	Asn	Leu	Trp	Ile	Thr	Cys	Gly		

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 467 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

Met 1	Ser	Leu	Ser	Leu 5	Trp	Gln	Gln	Cys	Leu 10	Ala	Arg	Leu	Gln	Asp 15	Glu
Leu	Pro	Ala	Thr 20	Glu	Phe	Ser	Met	Trp 25	Ile	Arg	Pro	Leu	Gln 30	Ala	Glu
Leu	Ser	Asp 35	Asn	Thr	Leu	Ala	Leu 40	Tyr	Ala	Pro	Asn	Arg 45	Phe	Val	Leu
Asp	Trp 50	Val	Arg	Asp	Lys	Tyr 55	Leu	Asn	Asn	Ile	Asn 60	Gly	Leu	Leu	Thr
Ser 65	Phe	Cys	Gly	Ala	Asp 70	Ala	Pro	Gln	Leu	Arg 75	Phe	Glu	Val	Gly	Thr 80
Lys	Pro	Val	Thr	Gln 85	Thr	Pro	Gln	Ala	Ala 90	Val	Thr	Ser	Asn	Val 95	Ala
Ala	Pro	Ala	Gln 100	Val	Ala	Gln	Thr	Gln 105	Pro	Gln	Arg	Ala	Ala 110	Pro	Ser
Thr	Arg	Ser 115	Gly	Trp	Asp	Asn	Val 120	Pro	Ala	Pro	Ala	Glu 125	Pro	Thr	Tyr
Arg	Ser 130	Asn	Val	Asn	Val	Lys 135	His	Thr	Phe	Asp	Asn 140	Phe	Val	Glu	Gly
Lys 145	Ser	Asn	Gln	Leu	Ala 150	Arg	Ala	Ala	Ala	Arg 155	Gln	Val	Ala	Asp	Asn 160
Pro	Gly	Gly	Ala	Tyr 165	Asn	Pro	Leu	Phe	Leu 170	Tyr	Gly	Gly	Thr	Gly 175	Leu
Gly	Lys	Thr	His 180	Leu	Leu	His	Ala	Val 185	Gly	Asn	Gly	Ile	Met 190	Ala	Arg
Lys	Pro	Asn 195	Ala	Lys	Val	Val	Tyr 200	Met	His	Ser	Glu	Arg 205	Phe	Val	Gln
Asp	Met 210	Val	Lys	Ala	Leu	Gln 215	Asn	Asn	Ala	Ile	Glu 220	Glu	Phe	Lys	Arg
Tyr 225	Tyr	Arg	Ser	Val	Asp 230	Ala	Leu	Leu	Ile	Asp 235	Asp	Ile	Gln	Phe	Phe 240
Ala	Asn	Lys	Glu	Arg	Ser	Gln	Glu	Glu	Phe	Phe	His	Thr	Phe	Asn	Ala

[illegible]

(i) SEQUENCE CHARACTERISTICS:

- (ii) MOLECULE TYPE: protein

Met 1	Asp	Thr	Asn	Asn 5	Asn	Ile	Glu	Lys	Glu 10	Ile	Leu	Ala	Leu	Val 15	Lys
Gln	Asn	Pro	Lys 20	Val	Ser	Leu	Ile	Glu 25	Tyr	Glu	Asn	Tyr	Phe 30	Ser	Gln
Leu	Lys	Tyr 35	Asn	Pro	Asn	Ala	Ser 40	Lys	Ser	Asp	Ile	Ala 45	Phe	Phe	Tyr
Ala	Pro 50	Asn	Gln	Val	Leu	Cys 55	Thr	Thr	Ile	Thr	Ala 60	Lys	Tyr	Gly	Ala
Leu 65	Leu	Lys	Glu	Ile 70	Leu	Ser	Gln	Asn	Lys	Val 75	Gly	Met	His	Leu	Ala 80
His	Ser	Val	Asp 85	Val	Arg	Ile	Glu	Val	Ala 90	Pro	Lys	Ile	Gln	Ile 95	Asn
Ala	Gln	Ser	Asn 100	Ile	Asn	Tyr	Lys	Ala 105	Ile	Lys	Thr	Ser	Val	Lys	Asp
Ser	Tyr	Thr 115	Phe	Glu	Asn	Phe	Val 120	Val	Gly	Ser	Cys	Asn 125	Asn	Thr	Val
Tyr	Glu 130	Ile	Ala	Lys	Lys	Val 135	Ala	Gln	Ser	Asp	Thr 140	Pro	Pro	Tyr	Asn
Pro 145	Val	Leu	Phe	Tyr	Gly 150	Gly	Thr	Gly	Leu	Gly 155	Lys	Thr	His	Ile	Leu 160
Asn	Ala	Ile	Gly	Asn	His	Ala	Leu	Glu	Lys	His	Lys	Lys	Val	Val	Leu

165								170					175				
Val	Thr	Ser	Glu 180	Asp	Phe	Leu	Thr	Asp 185	Phe	Leu	Lys	His	Leu 190	Asp	Asn		
Lys	Thr	Met 195	Asp	Ser	Phe	Lys	Ala 200	Lys	Tyr	Arg	His	Cys 205	Asp	Phe	Phe		
Leu	Leu 210	Asp	Asp	Ala	Gln	Phe 215	Leu	Gln	Gly	Lys	Pro 220	Lys	Leu	Glu	Glu		
Glu 225	Phe	Phe	His	Thr	Phe 230	Asn	Glu	Leu	His	Ala 235	Asn	Ser	Lys	Gln	Ile 240		
Val	Leu	Ile	Ser	Asp 245	Arg	Ser	Pro	Lys	Asn	Ile	Ala	Gly	Leu	Glu	Asp 255		
Arg	Leu	Lys	Ser 260	Arg	Phe	Glu	Trp	Gly 265	Ile	Thr	Ala	Lys	Val 270	Met	Pro		
Pro	Asp	Leu 275	Glu	Thr	Lys	Leu	Ser 280	Ile	Val	Lys	Gln	Lys 285	Cys	Gln	Leu		
Asn	Gln 290	Ile	Thr	Leu	Pro	Glu 295	Glu	Val	Met	Glu	Tyr 300	Ile	Ala	Gln	His		
Ile 305	Ser	Asp	Asn	Ile	Arg 310	Gln	Met	Glu	Gly	Ala 315	Ile	Ile	Lys	Ile	Ser 320		
Val	Asn	Ala	Asn	Leu 325	Met	Asn	Ala	Ser	Ile 330	Asp	Leu	Asn	Leu	Ala 335	Lys		
Thr	Val	Leu	Glu 340	Asp	Leu	Gln	Lys	Asp 345	His	Ala	Glu	Gly	Ser 350	Ser	Leu		
Glu	Asn	Ile 355	Leu	Leu	Ala	Val	Ala 360	Gln	Ser	Leu	Asn	Leu 365	Lys	Ser	Ser		
Glu 370	Ile	Lys	Val	Ser	Ser	Arg 375	Gln	Lys	Asn	Val	Ala 380	Leu	Ala	Arg	Lys		
Leu 385	Val	Val	Tyr	Phe	Ala 390	Arg	Leu	Tyr	Thr	Pro 395	Asn	Pro	Thr	Leu	Ser 400		
Leu	Ala	Gln	Phe	Leu 405	Asp	Leu	Lys	Asp	His 410	Ser	Ser	Ile	Ser	Lys 415	Met		
Tyr	Ser	Gly	Val 420	Lys	Lys	Met	Leu	Glu 425	Glu	Glu	Lys	Ser	Pro 430	Phe	Val		
Leu	Ser	Leu 435	Arg	Glu	Glu	Ile	Lys 440	Asn	Arg	Leu	Asn	Glu 445	Leu	Asn	Asp		
Lys	Lys 450	Thr	Ala	Phe	Asn	Ser	Ser	Glu									

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1305 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

GTGTCGCACG AGGCCGTCTG GCAACACGTT CTGGAGCACA TCCGCCGCAG CATCACCGAG 60
GTGGAGTTCC ACACCTGGTT TGAAAGGATC CGCCCCTTGG GGATCCGGA CGGGGTGCTG 120
GAGCTCGCCG TGCCACCTC CTTTGCCCTG GACTGGATCC GGCGCCACTA CGCCGGCCTC 180
ATCCAGGAGG GCCCTCGGCT CCTCGGGGCC CAGGCGCCCC GGTTTGAGCT CCGGGTGGTG 240
CCCGGGGTCG TAGTCCAGGA GGACATCTTC CAGCCCCCGC CGAGCCCCCC GGCCCAAGCT 300
CAACCCGAAG ATACCTTTAA AACTTCGTGG TGGGGCCCAA CAACTCCATG GCCCCACGGC 360
GGCGCCGTGG CCGTGCCGA GTCCCCCGGC CGGGCCTACA ACCCCCTCTT CATCTACGGG 420
GGCCGTGGCC TGGGAAAGAC CTACCTGATG CACGCCGTGG GCCCACTCCG TGCGAAGCGC 480
TTCCCCACA TGAGATTAGA GTACGTTTCC ACGAAACTT TCACCAACGA GCTCATCAAC 540
CGGCCATCCG CGAGGGACCG GATGACGGAG TTCCGGGAGC GGTACCGCTC CGTGGACCTC 600
CTGCTGGTGG ACGACGTCCA GTTCATCGCC GGAAAGGAGC GCACCCAGGA GGAGTTTTTC 660
CACACCTTCA ACGCCCTTTA CGAGGCCAC AAGCAGATCA TCCTCTCCTC CGACCGGCCG 720
CCCAAGGACA TCCTCACCTT GGAGGCGCGC CTGCGGAGCC GCTTTGAGTG GGGCTGATC 780
ACCGACAATC CAGCCCCGA CCTGGAACC CGGATCGCCA TCCTGAAGAT GAACGCCAGC 840
AGCGGGCCTG AGGATCCCGA GGACGCCCTG GAGTACATCG CCCGGCAGGT CACCTCCAAC 900
ATCCGGGAGT GGAAGGGGC CCTCATGCGG GCATCGCCTT TCGCCTCCCT CAACGGCGTT 960
GAGCTGACCC GCGCCGTGGC GGCCAAGGCT CTCCGACATC TTCGCCCCAG GGAGCTGGAG 1020
GCGGACCCTT TGAGATCAT CCGCAAAGCG GCGGGACCAG TTCGGCCTGA AACCCCGGA 1080
GGAGCTCACG GGGAGCGCCG CAAGAAGGAG GTGGTCCTCC CCCGGCAGCT CGCCATGTAC 1140
CTGGTGCGGG AGCTACCCC GGCTCCCTG CCCGAGATCG ACCAGCTCAA CGACGACCGG 1200
GACCACACCA CGGTCTCTA CGCCATCCAG AAGGTCCAGG AGCTCGCGGA AAGCGACCGG 1260
GAGGTGCAGG GCCTCTCCG CACCCTCCGG GAGGCGTGCA CATGA 1305

(2) INFORMATION FOR SEQ ID NO:105:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 434 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

Val Ser His Glu Ala Val Trp Gln His Val Leu Glu His Ile Arg Arg
1 5 10 15
Ser Ile Thr Glu Val Glu Phe His Thr Trp Phe Glu Arg Ile Arg Pro
20 25 30
Leu Gly Ile Arg Asp Gly Val Leu Glu Leu Ala Val Pro Thr Ser Phe
35 40 45
Ala Leu Asp Trp Ile Arg Arg His Tyr Ala Gly Leu Ile Gln Glu Gly
50 55 60
Pro Arg Leu Leu Gly Ala Gln Ala Pro Arg Phe Glu Leu Arg Val Val
65 70 75 80
Pro Gly Val Val Val Gln Glu Asp Ile Phe Gln Pro Pro Pro Ser Pro
85 90 95
Pro Ala Gln Ala Gln Pro Glu Asp Thr Phe Lys Thr Ser Trp Trp Gly
100 105 110
Pro Thr Thr Pro Trp Pro His Gly Gly Ala Val Ala Val Ala Glu Ser
115 120 125
Pro Gly Arg Ala Tyr Asn Pro Leu Phe Ile Tyr Gly Gly Arg Gly Leu
130 135 140
Gly Lys Thr Tyr Leu Met His Ala Val Gly Pro Leu Arg Ala Lys Arg
145 150 155 160
Phe Pro His Met Arg Leu Glu Tyr Val Ser Thr Glu Thr Phe Thr Asn
165 170 175
Glu Leu Ile Asn Arg Pro Ser Ala Arg Asp Arg Met Thr Glu Phe Arg
180 185 190
Glu Arg Tyr Arg Ser Val Asp Leu Leu Leu Val Asp Asp Val Gln Phe
195 200 205
Ile Ala Gly Lys Glu Arg Thr Gln Glu Glu Phe Phe His Thr Phe Asn
210 215 220
Ala Leu Tyr Glu Ala His Lys Gln Ile Ile Leu Ser Ser Asp Arg Pro
225 230 235 240
Pro Lys Asp Ile Leu Thr Leu Glu Ala Arg Leu Arg Ser Arg Phe Glu
245 250 255
Trp Gly Leu Ile Thr Asp Asn Pro Ala Pro Asp Leu Glu Thr Arg Ile
260 265 270
Ala Ile Leu Lys Met Asn Ala Ser Ser Gly Pro Glu Asp Pro Glu Asp
275 280 285
Ala Leu Glu Tyr Ile Ala Arg Gln Val Thr Ser Asn Ile Arg Glu Trp
290 295 300
Glu Gly Ala Leu Met Arg Ala Ser Pro Phe Ala Ser Leu Asn Gly Val
305 310 315 320

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Glu	Leu	Thr	Arg	Ala	Val	Ala	Ala	Lys	Ala	Leu	Arg	His	Leu	Arg	Pro		
				325					330					335			
Arg	Glu	Leu	Glu	Ala	Asp	Pro	Leu	Glu	Ile	Ile	Arg	Lys	Ala	Ala	Gly		
				340					345					350			
Pro	Val	Arg	Pro	Glu	Thr	Pro	Gly	Gly	Ala	His	Gly	Glu	Arg	Arg	Lys		
				355					360					365			
Lys	Glu	Val	Val	Leu	Pro	Arg	Gln	Leu	Ala	Met	Tyr	Leu	Val	Arg	Glu		
				370					375					380			
Leu	Thr	Pro	Ala	Ser	Leu	Pro	Glu	Ile	Asp	Gln	Leu	Asn	Asp	Asp	Arg		
385					390					395					400		
Asp	His	Thr	Thr	Val	Leu	Tyr	Ala	Ile	Gln	Lys	Val	Gln	Glu	Leu	Ala		
				405					410					415			
Glu	Ser	Asp	Arg	Glu	Val	Gln	Gly	Leu	Leu	Arg	Thr	Leu	Arg	Glu	Ala		
				420					425					430			
Cys	Thr																

(2) INFORMATION FOR SEQ ID NO:106:

(ii) MOLECULE TYPE: cDNA

ATGAACATAA	CGGTTCCCAA	AAAACCTCCTC	TCGGACCAGC	TTTCCCTCCT	GGAGCGCATC	60
GTCCCCCTCTA	GAAGCGCCAA	CCCCCTCTAC	ACCTACCTGG	GGCTTTACGC	CGAGGAAGGG	120
GCCTTGATCC	TCTTCGGGAC	CAACGGGGAG	GTGGACCTCG	AGGTCCGCCT	CCCCGCCGAG	180
GCCCAAAGCC	TTCCCCGGGT	GCTCGTCCCC	GCCCAGCCCT	TCTTCCAGCT	GGTGCGGAGC	240
CTTCCTGGGG	ACCTCGTGGC	CCTCGGCCTC	GCCTCGGAGC	CGGGCCAGGG	GGGGCAGCTG	300
GAGCTCTCCT	CCGGGCGTTT	CCGCACCCGG	CTCAGCCTGG	CCCCTGCCGA	GGGCTACCCC	360
GAGCTTCTGG	TGCCCCGAGG	GGAGGACAAG	GGGGCCTTCC	CCCTCCGGAC	GCGGATGCCC	420
TCCGGGGAGC	TCGTCAAGGC	CTTGACCCAC	GTGCGCTACG	CCGCAGCAA	CGAGGAGTAC	480
CGGGCCATCT	TCCGCGGGGT	GCAGCTGGAG	TTCTCCCCC	AGGGCTTCCG	GGCGGTGGCC	540
TCCGACGGGT	ACCGCCTCGC	CCTCTACGAC	CTGCCCTGC	CCCAAGGGTT	CCAGGCCAAG	600
GCCGTGGTCC	CCGCCCGAG	CGTGGACGAG	ATGGTGCGGG	TCCTGAAGGG	GGCGGACGGG	660
GCCGAGGCCG	TCCTCGCCCT	GGGCGAGGGG	GTGTTGGCCC	TGGCCCTCGA	GGGCGGAAGC	720

GGGGTCCGGA TGGCCCTCCG CCTCATGGAA GGGGAGTTCC CCGACTACCA GAGGGTCATC 780
 CCCCAGGAGT TCGCCCTCAA GGTCCAGGTG GAGGGGGAGG CCCTCAGGGA GGCGGTGCGC 840
 CGGGTGAGCG TCCTCTCCGA CCGGCAGAAC CACCGGGTGG ACCTCCTTTT GGAGGAAGGC 900
 CGGATCCTCC TCTCCGCCGA GGGGGACTAC GGCAAGGGGC AGGAGGAGGT GCCCGCCCAG 960
 GTGGAGGGGC CGGACATGGC CGTGGCCTAC AACGCCCCGT ACCTCCTCGA GGCCCTCGCC 1020
 CCCGTGGGGG ACCGGGCCCCA CCTGGGCATC TCCGGGCCCCA CGAGCCCGAG CCTCATCTGG 1080
 GGGGACGGGG AGGGGTACCG GGCGGTGGTG GTGCCCCCTCA GGGTCTAG 1128

(2) INFORMATION FOR SEQ ID NO:107:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 376 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

Met	Asn	Ile	Thr	Val	Pro	Lys	Lys	Leu	Leu	Ser	Asp	Gln	Leu	Ser	Leu
1				5				10						15	
Leu	Glu	Arg	Ile	Val	Pro	Ser	Arg	Ser	Ala	Asn	Pro	Leu	Tyr	Thr	Tyr
			20					25					30		
Leu	Gly	Leu	Tyr	Ala	Glu	Glu	Gly	Ala	Leu	Ile	Leu	Phe	Gly	Thr	Asn
		35					40					45			
Gly	Glu	Val	Asp	Leu	Glu	Val	Arg	Leu	Pro	Ala	Glu	Ala	Gln	Ser	Leu
	50					55					60				
Pro	Arg	Val	Leu	Val	Pro	Ala	Gln	Pro	Phe	Phe	Gln	Leu	Val	Arg	Ser
65				70					75					80	
Leu	Pro	Gly	Asp	Leu	Val	Ala	Leu	Gly	Leu	Ala	Ser	Glu	Pro	Gly	Gln
			85					90						95	
Gly	Gly	Gln	Leu	Glu	Leu	Ser	Ser	Gly	Arg	Phe	Arg	Thr	Arg	Leu	Ser
		100						105					110		
Leu	Ala	Pro	Ala	Glu	Gly	Tyr	Pro	Glu	Leu	Leu	Val	Pro	Glu	Gly	Glu
	115					120						125			
Asp	Lys	Gly	Ala	Phe	Pro	Leu	Arg	Thr	Arg	Met	Pro	Ser	Gly	Glu	Leu
	130					135					140				
Val	Lys	Ala	Leu	Thr	His	Val	Arg	Tyr	Ala	Ala	Ser	Asn	Glu	Glu	Tyr
145				150					155					160	
Arg	Ala	Ile	Phe	Arg	Gly	Val	Gln	Leu	Glu	Phe	Ser	Pro	Gln	Gly	Phe
			165					170						175	

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Arg Ala Val Ala Ser Asp Gly Tyr Arg Leu Ala Leu Tyr Asp Leu Pro
 180 185 190
 Leu Pro Gln Gly Phe Gln Ala Lys Ala Val Val Pro Ala Arg Ser Val
 195 200 205
 Asp Glu Met Val Arg Val Leu Lys Gly Ala Asp Gly Ala Glu Ala Val
 210 215 220
 Leu Ala Leu Gly Glu Gly Val Leu Ala Leu Ala Leu Glu Gly Gly Ser
 225 230 235 240
 Gly Val Arg Met Ala Leu Arg Leu Met Glu Gly Glu Phe Pro Asp Tyr
 245 250 255
 Gln Arg Val Ile Pro Gln Glu Phe Ala Leu Lys Val Gln Val Glu Gly
 260 265 270
 Glu Ala Leu Arg Glu Ala Val Arg Arg Val Ser Val Leu Ser Asp Arg
 275 280 285
 Gln Asn His Arg Val Asp Leu Leu Leu Glu Glu Gly Arg Ile Leu Leu
 290 295 300
 Ser Ala Glu Gly Asp Tyr Gly Lys Gly Gln Glu Glu Val Pro Ala Gln
 305 310 315 320
 Val Glu Gly Pro Asp Met Ala Val Ala Tyr Asn Ala Arg Tyr Leu Leu
 325 330 335
 Glu Ala Leu Ala Pro Val Gly Asp Arg Ala His Leu Gly Ile Ser Gly
 340 345 350
 Pro Thr Ser Pro Ser Leu Ile Trp Gly Asp Gly Glu Gly Tyr Arg Ala
 355 360 365
 Val Val Val Pro Leu Arg Val Glx
 370 375

(2) INFORMATION FOR SEQ ID NO:108:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 376 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

Met Asn Ile Thr Val Pro Lys Lys Leu Leu Ser Asp Gln Leu Ser Leu
 1 5 10 15
 Leu Glu Arg Ile Val Pro Ser Arg Ser Ala Asn Pro Leu Tyr Thr Tyr
 20 25 30
 Leu Gly Leu Tyr Ala Glu Glu Gly Ala Leu Ile Leu Phe Gly Thr Asn
 35 40 45

Gly 50	Glu	Val	Asp	Leu	Glu	Val	Arg	Leu	Pro	Ala	Glu	Ala	Gln	Ser	Leu
Pro 65	Arg	Val	Leu	Val	Pro	Ala	Gln	Pro	Phe	Phe	Gln	Leu	Val	Arg	Ser
Leu	Pro	Gly	Asp	Leu	Val	Ala	Leu	Gly	Leu	Ala	Ser	Glu	Pro	Gly	Gln
Gly	Gly	Gln	Leu	Glu	Leu	Ser	Ser	Gly	Arg	Phe	Arg	Thr	Arg	Leu	Ser
Leu	Ala	Pro	Ala	Glu	Gly	Tyr	Pro	Glu	Leu	Leu	Val	Pro	Glu	Gly	Glu
Asp	Lys	Gly	Ala	Phe	Pro	Leu	Arg	Thr	Arg	Met	Pro	Ser	Gly	Glu	Leu
Val	Lys	Ala	Leu	Thr	His	Val	Arg	Tyr	Ala	Ala	Ser	Asn	Glu	Glu	Tyr
Arg	Ala	Ile	Phe	Arg	Gly	Val	Gln	Leu	Glu	Phe	Ser	Pro	Gln	Gly	Phe
Arg	Ala	Val	Ala	Ser	Asp	Gly	Tyr	Arg	Leu	Ala	Leu	Tyr	Asp	Leu	Pro
Leu	Pro	Gln	Gly	Phe	Gln	Ala	Lys	Ala	Val	Val	Pro	Ala	Arg	Ser	Val
Asp	Glu	Met	Val	Arg	Val	Leu	Lys	Gly	Ala	Asp	Gly	Ala	Glu	Ala	Val
Leu	Ala	Leu	Gly	Glu	Gly	Val	Leu	Ala	Leu	Ala	Leu	Glu	Gly	Gly	Ser
Gly	Val	Arg	Met	Ala	Leu	Arg	Leu	Met	Glu	Gly	Glu	Phe	Pro	Asp	Tyr
Gln	Arg	Val	Ile	Pro	Gln	Glu	Phe	Ala	Leu	Lys	Val	Gln	Val	Glu	Gly
Glu	Ala	Leu	Arg	Glu	Ala	Val	Arg	Arg	Val	Ser	Val	Leu	Ser	Asp	Arg
Gln	Asn	His	Arg	Val	Asp	Leu	Leu	Leu	Glu	Glu	Gly	Arg	Ile	Leu	Leu
Ser	Ala	Glu	Gly	Asp	Tyr	Gly	Lys	Gly	Gln	Glu	Glu	Val	Pro	Ala	Gln
Val	Glu	Gly	Pro	Asp	Met	Ala	Val	Ala	Tyr	Asn	Ala	Arg	Tyr	Leu	Leu
Glu	Ala	Leu	Ala	Pro	Val	Gly	Asp	Arg	Ala	His	Leu	Gly	Ile	Ser	Gly
Pro	Thr	Ser	Pro	Ser	Leu	Ile	Trp	Gly	Asp	Gly	Glu	Gly	Tyr	Arg	Ala
Val	Val	Val	Pro	Leu	Arg	Val	Glx								

1660 1670 1680 1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
 1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920 1930 1940 1950
 1960 1970 1980 1990 2000 2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
 2110 2120 2130 2140 2150 2160 2170 2180 2190 2200 2210 2220 2230 2240 2250
 2260 2270 2280 2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
 2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520 2530 2540 2550
 2560 2570 2580 2590 2600 2610 2620 2630 2640 2650 2660 2670 2680 2690 2700
 2710 2720 2730 2740 2750 2760 2770 2780 2790 2800 2810 2820 2830 2840 2850
 2860 2870 2880 2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
 3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120 3130 3140 3150
 3160 3170 3180 3190 3200 3210 3220 3230 3240 3250 3260 3270 3280 3290 3300
 3310 3320 3330 3340 3350 3360 3370 3380 3390 3400 3410 3420 3430 3440 3450
 3460 3470 3480 3490 3500 3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
 3610 3620 3630 3640 3650 3660 3670 3680 3690 3700 3710 3720 3730 3740 3750
 3760 3770 3780 3790 3800 3810 3820 3830 3840 3850 3860 3870 3880 3890 3900
 3910 3920 3930 3940 3950 3960 3970 3980 3990 4000 4010 4020 4030 4040 4050
 4060 4070 4080 4090 4100 4110 4120 4130 4140 4150 4160 4170 4180 4190 4200
 4210 4220 4230 4240 4250 4260 4270 4280 4290 4300 4310 4320 4330 4340 4350
 4360 4370 4380 4390 4400 4410 4420 4430 4440 4450 4460 4470 4480 4490 4500
 4510 4520 4530 4540 4550 4560 4570 4580 4590 4600 4610 4620 4630 4640 4650
 4660 4670 4680 4690 4700 4710 4720 4730 4740 4750 4760 4770 4780 4790 4800
 4810 4820 4830 4840 4850 4860 4870 4880 4890 4900 4910 4920 4930 4940 4950
 4960 4970 4980 4990 5000 5010 5020 5030 5040 5050 5060 5070 5080 5090 5100
 5110 5120 5130 5140 5150 5160 5170 5180 5190 5200 5210 5220 5230 5240 5250
 5260 5270 5280 5290 5300 5310 5320 5330 5340 5350 5360 5370 5380 5390 5400
 5410 5420 5430 5440 5450 5460 5470 5480 5490 5500 5510 5520 5530 5540 5550
 5560 5570 5580 5590 5600 5610 5620 5630 5640 5650 5660 5670 5680 5690 5700
 5710 5720 5730 5740 5750 5760 5770 5780 5790 5800 5810 5820 5830 5840 5850
 5860 5870 5880 5890 5900 5910 5920 5930 5940 5950 5960 5970 5980 5990 6000
 6010 6020 6030 6040 6050 6060 6070 6080 6090 6100 6110 6120 6130 6140 6150
 6160 6170 6180 6190 6200 6210 6220 6230 6240 6250 6260 6270 6280 6290 6300
 6310 6320 6330 6340 6350 6360 6370 6380 6390 6400 6410 6420 6430 6440 6450
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 6610 6620 6630 6640 6650 6660 6670 6680 6690 6700 6710 6720 6730 6740 6750
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 6910 6920 6930 6940 6950 6960 6970 6980 6990 7000 7010 7020 7030 7040 7050
 7060 7070 7080 7090 7100 7110 7120 7130 7140 7150 7160 7170 7180 7190 7200
 7210 7220 7230 7240 7250 7260 7270 7280 7290 7300 7310 7320 7330 7340 7350
 7360 7370 7380 7390 7400 7410 7420 7430 7440 7450 7460 7470 7480 7490 7500
 7510 7520 7530 7540 7550 7560 7570 7580 7590 7600 7610 7620 7630 7640 7650
 7660 7670 7680 7690 7700 7710 7720 7730 7740 7750 7760 7770 7780 7790 7800
 7810 7820 7830 7840 7850 7860 7870 7880 7890 7900 7910 7920 7930 7940 7950
 7960 7970 7980 7990 8000 8010 8020 8030 8040 8050 8060 8070 8080 8090 8100
 8110 8120 8130 8140 8150 8160 8170 8180 8190 8200 8210 8220 8230 8240 8250
 8260 8270 8280 8290 8300 8310 8320 8330 8340 8350 8360 8370 8380 8390 8400
 8410 8420 8430 8440 8450 8460 8470 8480 8490 8500 8510 8520 8530 8540 8550
 8560 8570 8580 8590 8600 8610 8620 8630 8640 8650 8660 8670 8680 8690 8700
 8710 8720 8730 8740 8750 8760 8770 8780 8790 8800 8810 8820 8830 8840 8850
 8860 8870 8880 8890 8900 8910 8920 8930 8940 8950 8960 8970 8980 8990 9000
 9010 9020 9030 9040 9050 9060 9070 9080 9090 9100 9110 9120 9130 9140 9150
 9160 9170 9180 9190 9200 9210 9220 9230 9240 9250 9260 9270 9280 9290 9300
 9310 9320 9330 9340 9350 9360 9370 9380 9390 9400 9410 9420 94

[illegible]

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(17) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 367 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

Met 1	Lys	Phe	Thr	Val 5	Glu	Arg	Glu	His 10	Leu	Lys	Pro	Leu	Gln 15	Gln	
Val	Ser	Gly	Pro 20	Leu	Gly	Gly	Arg	Pro 25	Thr	Leu	Pro	Ile	Leu 30	Gly	Asn
Leu	Leu	Leu 35	Gln	Val	Ala	Asp	Gly 40	Thr	Leu	Ser	Leu	Thr 45	Gly	Thr	Asp
Leu	Glu 50	Met	Glu	Met	Val	Ala 55	Arg	Val	Ala	Leu	Val 60	Gln	Pro	His	Glu
Pro 65	Gly	Ala	Thr	Thr 70	Val	Pro	Ala	Arg	Lys	Phe 75	Phe	Asp	Ile	Cys	Arg 80
Gly	Leu	Pro	Glu	Gly 85	Ala	Glu	Ile	Ala	Val 90	Gln	Leu	Glu	Gly	Glu 95	Arg
Met	Leu	Val 100	Arg	Ser	Gly	Arg	Ser	Arg 105	Phe	Ser	Leu	Ser 110	Thr	Leu	Pro
Ala	Ala	Asp 115	Phe	Pro	Asn	Leu	Asp 120	Asp	Trp	Gln	Ser	Glu 125	Val	Glu	Phe
Thr 130	Leu	Pro	Gln	Ala	Thr	Met 135	Lys	Arg	Leu	Ile 140	Glu	Ala	Thr	Gln	Phe
Ser 145	Met	Ala	His	Gln	Asp 150	Val	Arg	Tyr	Tyr	Leu 155	Asn	Gly	Met	Leu	Phe 160
Glu	Thr	Glu	Gly	Glu 165	Glu	Leu	Arg	Thr	Val 170	Ala	Thr	Asp	Gly	His 175	Arg
Leu	Ala	Val 180	Cys	Ser	Met	Pro	Ile	Gly 185	Gln	Ser	Leu	Pro 190	Ser	His	Ser
Val 195	Ile	Val	Pro	Arg	Lys	Gly	Val 200	Ile	Glu	Leu	Met	Arg 205	Met	Leu	Asp
Gly 210	Gly	Asp	Asn	Pro	Leu	Arg 215	Val	Gln	Ile	Gly	Ser 220	Asn	Asn	Ile	Arg
Ala 225	His	Val	Gly	Asp	Phe 230	Ile	Phe	Thr	Ser	Lys 235	Leu	Val	Asp	Gly	Arg 240
Phe	Pro	Asp	Tyr	Arg 245	Arg	Val	Leu	Pro	Lys 250	Asn	Pro	Asp	Lys	His 255	Leu

Glu Ala Gly Cys Asp Leu Leu Lys Gln Ala Phe Ala Arg Ala Ala Ile
 260 265 270
 Leu Ser Asn Glu Lys Phe Arg Gly Val Arg Leu Tyr Val Ser Glu Asn
 275 280 285
 Gln Leu Lys Ile Thr Ala Asn Asn Pro Glu Gln Glu Glu Ala Glu Glu
 290 295 300
 Ile Leu Asp Val Thr Tyr Ser Gly Ala Glu Met Glu Ile Gly Phe Asn
 305 310 315 320
 Val Ser Tyr Val Leu Asp Val Leu Asn Ala Leu Lys Cys Glu Asn Val
 325 330 335
 Arg Met Met Leu Thr Asp Ser Val Ser Ser Val Gln Ile Glu Asp Ala
 340 345 350
 Ala Ser Gln Ser Ala Ala Tyr Val Val Met Pro Met Arg Leu Glx
 355 360 365

(2) INFORMATION FOR SEQ ID NO:110:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 367 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

Met Lys Phe Ile Ile Glu Arg Glu Gln Leu Leu Lys Pro Leu Gln Gln
 1 5 10 15
 Val Ser Gly Pro Leu Gly Gly Arg Pro Thr Leu Pro Ile Leu Gly Asn
 20 25 30
 Leu Leu Leu Lys Val Thr Glu Asn Thr Leu Ser Leu Thr Gly Thr Asp
 35 40 45
 Leu Glu Met Glu Met Met Ala Arg Val Ser Leu Ser Gln Ser His Glu
 50 55 60
 Ile Gly Ala Thr Thr Val Pro Ala Arg Lys Phe Phe Asp Ile Trp Arg
 65 70 75 80
 Gly Leu Pro Glu Gly Ala Glu Ile Ser Val Glu Leu Asp Gly Asp Arg
 85 90 95
 Leu Leu Val Arg Ser Gly Arg Ser Arg Phe Ser Leu Ser Thr Leu Pro
 100 105 110
 Ala Ser Asp Phe Pro Asn Leu Asp Asp Trp Gln Ser Glu Val Glu Phe
 115 120 125
 Thr Leu Pro Gln Ala Thr Leu Lys Arg Leu Ile Glu Ser Thr Gln Phe
 130 135 140

Ser Met Ala His Gln Asp Val Arg Tyr Tyr Leu Asn Gly Met Leu Phe
 145 150 155 160
 Glu Thr Glu Asn Thr Glu Leu Arg Thr Val Ala Thr Asp Gly His Arg
 165 170 175
 Leu Ala Val Cys Ala Met Asp Ile Gly Gln Ser Leu Pro Gly His Ser
 180 185 190
 Val Ile Val Pro Arg Lys Gly Val Ile Glu Leu Met Arg Leu Leu Asp
 195 200 205
 Gly Ser Gly Glu Ser Leu Leu Gln Leu Gln Ile Gly Ser Asn Asn Leu
 210 215 220
 Arg Ala His Val Gly Asp Phe Ile Phe Thr Ser Lys Leu Val Asp Gly
 225 230 235 240
 Arg Phe Pro Asp Tyr Arg Arg Val Leu Pro Lys Asn Pro Thr Lys Thr
 245 250 255
 Val Ile Ala Gly Cys Asp Ile Leu Lys Gln Ala Phe Ser Arg Ala Ala
 260 265 270
 Ile Leu Ser Asn Glu Lys Phe Arg Gly Val Arg Ile Asn Leu Thr Asn
 275 280 285
 Gly Gln Leu Lys Ile Thr Ala Asn Asn Pro Glu Gln Glu Glu Ala Glu
 290 295 300
 Glu Ile Val Asp Val Gln Tyr Gln Gly Glu Glu Met Glu Ile Gly Phe
 305 310 315 320
 Asn Val Ser Tyr Leu Leu Asp Val Leu Asn Thr Leu Lys Cys Glu Glu
 325 330 335
 Val Lys Leu Leu Leu Thr Asp Ala Val Ser Ser Val Gln Val Glu Asn
 340 345 350
 Val Ala Ser Ala Ala Ala Ala Tyr Val Val Met Pro Met Arg Leu
 355 360 365

(2) INFORMATION FOR SEQ ID NO:111:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 366 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

Met Gln Phe Ser Ile Ser Arg Glu Asn Leu Leu Lys Pro Leu Gln Gln
 1 5 10 15
 Val Cys Gly Val Leu Ser Asn Arg Pro Asn Ile Pro Val Leu Asn Asn
 20 25 30

Val Leu Leu Gln Ile Glu Asp Tyr Arg Leu Thr Ile Thr Gly Thr Asp
 35 40 45
 Leu Glu Val Glu Leu Ser Ser Gln Thr Gln Leu Ser Ser Ser Ser Glu
 50 55 60
 Asn Gly Thr Phe Thr Ile Pro Ala Lys Lys Phe Leu Asp Ile Cys Arg
 65 70 75 80
 Thr Leu Ser Asp Asp Ser Glu Ile Thr Val Thr Phe Glu Gln Asp Arg
 85 90 95
 Ala Leu Val Gln Ser Gly Arg Ser Arg Phe Thr Leu Ala Thr Gln Pro
 100 105 110
 Ala Glu Glu Tyr Pro Asn Leu Thr Asp Trp Gln Ser Glu Val Asp Phe
 115 120 125
 Glu Leu Pro Gln Asn Thr Leu Arg Arg Leu Ile Glu Ala Thr Gln Phe
 130 135 140
 Ser Met Ala Asn Gln Asp Ala Arg Tyr Phe Leu Asn Gly Met Lys Phe
 145 150 155 160
 Glu Thr Glu Gly Asn Leu Leu Arg Thr Val Ala Thr Asp Gly His Arg
 165 170 175
 Leu Ala Val Cys Thr Ile Ser Leu Glu Gln Glu Leu Gln Asn His Ser
 180 185 190
 Val Ile Leu Pro Arg Lys Gly Val Leu Glu Leu Val Arg Leu Leu Glu
 195 200 205
 Thr Asn Asp Glu Pro Ala Arg Leu Gln Ile Gly Thr Asn Asn Leu Arg
 210 215 220
 Val His Leu Lys Asn Thr Val Phe Thr Ser Lys Leu Ile Asp Gly Arg
 225 230 235 240
 Phe Pro Asp Tyr Arg Arg Val Leu Pro Arg Asn Ala Thr Lys Ile Val
 245 250 255
 Glu Gly Asn Trp Glu Met Leu Lys Gln Ala Phe Ala Arg Ala Ser Ile
 260 265 270
 Leu Ser Asn Glu Arg Ala Arg Ser Val Arg Leu Ser Leu Lys Glu Asn
 275 280 285
 Gln Leu Lys Ile Thr Ala Ser Asn Thr Glu His Glu Glu Ala Glu Glu
 290 295 300
 Ile Val Asp Val Asn Tyr Asn Gly Glu Glu Leu Glu Val Gly Phe Asn
 305 310 315 320
 Val Thr Tyr Ile Leu Asp Val Leu Asn Ala Leu Lys Cys Asn Gln Val
 325 330 335
 Arg Met Cys Leu Thr Asp Ala Phe Ser Ser Cys Leu Ile Glu Asn Cys
 340 345 350
 Glu Asp Ser Ser Cys Glu Tyr Val Ile Met Pro Met Arg Leu
 355 360 365

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(2) INFORMATION FOR SEQ ID NO:112:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 367 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

Met His Phe Thr Ile Gln Arg Glu Ala Leu Leu Lys Pro Leu Gln Leu
1 5 10 15
Val Ala Gly Val Val Glu Arg Arg Gln Thr Leu Pro Val Leu Ser Asn
20 25 30
Val Leu Leu Val Val Gln Gly Gln Gln Leu Ser Leu Thr Gly Thr Asp
35 40 45
Leu Glu Val Glu Leu Val Gly Arg Val Gln Leu Glu Glu Pro Ala Glu
50 55 60
Pro Gly Glu Ile Thr Val Pro Ala Arg Lys Leu Met Asp Ile Cys Lys
65 70 75 80
Ser Leu Pro Asn Asp Ala Leu Ile Asp Ile Lys Val Asp Glu Gln Lys
85 90 95
Leu Leu Val Lys Ala Gly Arg Ser Arg Phe Thr Leu Ser Thr Leu Pro
100 105 110
Ala Asn Asp Phe Pro Thr Val Glu Glu Gly Pro Gly Ser Leu Thr Cys
115 120 125
Asn Leu Glu Gln Ser Lys Leu Arg Arg Leu Ile Glu Arg Thr Ser Phe
130 135 140
Ala Met Ala Gln Gln Asp Val Arg Tyr Tyr Leu Asn Gly Met Leu Leu
145 150 155 160
Glu Val Ser Arg Asn Thr Leu Arg Ala Val Ser Thr Asp Gly His Arg
165 170 175
Leu Ala Leu Cys Ser Met Ser Ala Pro Ile Glu Gln Glu Asp Arg His
180 185 190
Gln Val Ile Val Pro Arg Lys Gly Ile Leu Glu Leu Ala Arg Leu Leu
195 200 205
Thr Asp Pro Glu Gly Met Val Ser Ile Val Leu Gly Gln His His Ile
210 215 220
Arg Ala Thr Thr Gly Glu Phe Thr Phe Thr Ser Lys Leu Val Asp Gly
225 230 235 240
Lys Phe Pro Asp Tyr Glu Arg Val Leu Pro Lys Gly Gly Asp Lys Leu
245 250 255

Ser Met Gly Lys Gln Asp Val Arg Tyr Tyr Leu Asn Gly Met Leu Leu
 145 150 155 160
 Glu Lys Lys Asp Lys Phe Leu Arg Ser Val Ala Thr Asp Gly Tyr Arg
 165 170 175
 Leu Ala Ile Ser Tyr Thr Gln Leu Lys Lys Asp Ile Asn Phe Phe Ser
 180 185 190
 Ile Ile Ile Pro Asn Lys Ala Val Met Glu Leu Leu Lys Leu Leu Asn
 195 200 205
 Thr Gln Pro Gln Leu Leu Asn Ile Leu Ile Gly Ser Asn Ser Ile Arg
 210 215 220
 Ile Tyr Thr Lys Asn Leu Ile Phe Thr Thr Gln Leu Ile Glu Gly Glu
 225 230 235 240
 Tyr Pro Asp Tyr Lys Ser Val Leu Phe Lys Glu Lys Lys Asn Pro Ile
 245 250 255
 Ile Thr Asn Ser Ile Leu Leu Lys Lys Ser Leu Leu Arg Val Ala Ile
 260 265 270
 Leu Ala His Glu Lys Phe Cys Gly Ile Glu Ile Lys Ile Glu Asn Gly
 275 280 285
 Lys Phe Lys Val Leu Ser Asp Asn Gln Glu Glu Glu Thr Ala Glu Asp
 290 295 300
 Leu Phe Glu Ile Asp Tyr Phe Gly Glu Lys Ile Glu Ile Ser Ile Asn
 305 310 315 320
 Val Tyr Tyr Leu Leu Asp Val Ile Asn Asn Ile Lys Ser Glu Asn Ile
 325 330 335
 Ala Leu Phe Leu Asn Lys Ser Lys Ser Ser Ile Gln Ile Glu Ala Glu
 340 345 350
 Asn Asn Ser Ser Asn Ala Tyr Val Val Met Leu Leu Lys Arg
 355 360 365

(2) INFORMATION FOR SEQ ID NO:114:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PRIMER"
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

GTGTGGATCC TCGTCCCCCT CATGCGCGAC CAGGAAGGG

39

(2) INFORMATION FOR SEQ ID NO:115:

Figure 1 is a schematic representation of the experimental design. It shows a flow from 'Study 1' to 'Study 2'. Study 1 involves 'Pretest' and 'Main Study'. Study 2 involves 'Pretest' and 'Main Study'. The 'Main Study' in Study 2 is further divided into 'Control' and 'Intervention' groups. The 'Intervention' group is further divided into 'Intervention 1' and 'Intervention 2'.

(iii) HYPOTHETICAL: NO

GTGTGGATCC GTGGTGACCT TAGCCAC

27

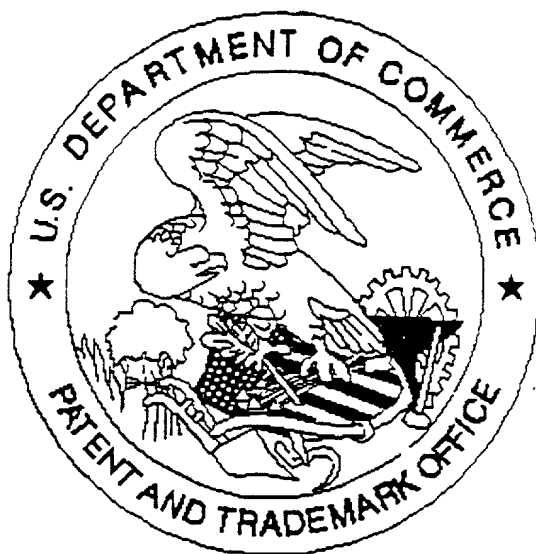
(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

TTCGTGTCCG AGGACCTTGT GGTCCACAAC

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